Treatment of localised cutaneous *Leishmania tropica* infection in Aleppo, Syria and drug sensitivity of clinical isolates

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I, Nizar Abazid, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Anthroponotic cutaneous leishmaniasis caused by *Leishmania tropica* has been endemic in Aleppo, Syria for centuries. The first modern description of the disease was also done in Aleppo. A surveillance system is in place, and the numbers of annual recorded cases have been rising from a few hundred to thousands in the late 1980s, to more than 5,000 in most years from 1990, and to more than 10,000 since 2003.

A retrospective analysis of routinely collected demographic data was performed. The clinical course was examined in a subset of patients. One hundred and thirty-two patients were recruited for follow-up study. Parasites were isolated from the lesions of these patients before treatment and during the course of treatment. Eighty isolates were tested for drug sensitivity in amastigote–macrophage system and typed to species level. Molecular fingerprinting was applied to a subset of isolates. Interviews were held with patients or accompanying adults about their knowledge, attitudes and practices regarding prevention, diagnosis and treatment.

Leishmaniasis patients in Aleppo were younger than the general population (median age 13 *vs.* 19 years), and females predominated among adults. Children and males were more likely to have lesions on the face. Smear positivity decreased with patient age (OR=0.5 in over-forties compared to under-tens). Smear positivity peaked at two-month lesion duration (OR=2.2 compared to lesion duration of <1 month). A significant proportion of patients, especially adults, did not complete their treatment course.

The isolated parasites were insensitive (median EC_{50}=229 μg Sb^V/ml) to pentavalent antimony, the drug used in Aleppo, and to paromomycin but were sensitive to amphotericin B. No relationship was found between baseline parasite *in vitro* sensitivity and treatment duration. All the typed parasites were *L. tropica*. Parasite schizodemes clustered by place of isolation and by family.

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Acronyms and abbreviations

ACL	anthroponotic cutaneous leishmaniasis
AmB	amphotericin B
CCL	chronic cutaneous leishmaniasis
CL	cutaneous leishmaniasis
DCL	diffuse (disseminated) cutaneous leishmaniasis
DMSO	dimethyl sulphoxide
EC_{50}	fifty-percent effective concentration
fPPG	filamentous proteophosphoglycan
HIFCS	heat-inactivated foetal calf serum
HIV	human immunodeficiency virus
IFN-γ	interferon-γ
IL	interleukin
i.l.	intralesional
i.m.	intramuscular
i.v.	intravenous
K27	the reference strain of *Leishmania tropica*, MHOM/SU/74/K27
KAP	knowledge, attitudes and practices
kDa	kilodalton
LCC	Leishmaniasis Control Center (Aleppo)
LCL	localised cutaneous leishmaniasis
MCL	mucocutaneous leishmaniasis
MGA	meglumine antimoniate
MLEE	multilocus enzyme electrophoresis
MoD	Ministry of Defence (Syria)
MoH	Ministry of Health (Syria)
MSF	Médecins Sans Frontières
PBS	phosphate-buffered saline
PHC	Primary health care
PKDL	post-kala-azar dermal leishmaniasis
PSG	promastigote secretory gel
Sb^{III}	trivalent antimony preparation(s), *or, in drug assays,* potassium antimony(III) oxide tartrate hemihydrate
Sb^{V}	Pentavalent antimony preparation(s)
SSG	sodium stibogluconate
TGF-β	transforming growth factor β
TNF-α	tumour necrosis factor α
WHO	World Health Organization
ZVL	zoonotic visceral leishmaniasis

Chapter 1. Introduction

1.1. Leishmaniases: overview and epidemiology

The leishmaniases are a group of parasitic diseases caused by obligate intracellular protozoan parasites of the genus *Leishmania* [Herwaldt, 1999] and transmitted by sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Leishmaniases are endemic in 88 countries in tropical and subtropical zones of all inhabited continents except Australia. [Desjeux, 2001; Blum *et al.*, 2004; World Health Organization, 2004].

About 20 species of *Leishmania* are known to cause disease in man (Table 1.1). Most of them are originally zoonotic, affecting mainly small rodents that act as an animal reservoir.

Four major eco-epidemiologic entities are recognised: zoonotic and anthroponotic visceral leishmaniasis, or kala-azar, and zoonotic and anthroponotic cutaneous leishmaniasis [Desjeux, 2001].

No reliable data on worldwide leishmaniasis prevalence and incidence exist because leishmaniasis frequently occurs in under-served rural areas and is not diagnosed; in addition, even diagnosed cases are under-reported because leishmaniasis is notifiable in only 33 of the 88 endemic countries. Due to this, its occurrence can only be estimated. Conducted surveys always showed higher prevalence than thought previously [World Health Organization, 2006].

The annual incidence is estimated at about 2 million cases worldwide, 500,000 for visceral and 1,500,000 for cutaneous forms; overall prevalence is about 12 million, with 350 million at risk [Desjeux, 2001]. These estimates are based on the number of reported cases, and population at risk was estimated by calculating the "relevant population", which was defined as susceptible age groups in relevant (urban or rural) epidemiological setting multiplied by

proportion of country area where leishmaniasis is known to occur [Ashford *et al.*, 1992]. These estimates are very approximate and rather old, but these figures are cited by all papers, including the most recent ones, with the exception of one WHO publication [World Health Organization, 2007] which stated the number of cutaneous cases to be one million a year. However, because this is an information brochure, no references to support this number are mentioned.

1.1.1. The parasite

The first accurate description of *Leishmania* was published in 1898 by Borovsky who described oval bodies filling human cells in sections of cutaneous ulcers [Borovsky, 1898]. Borovsky classified them as Protozoa. In 1903 J. Wright discovered similar organisms in a patient from Armenia and suggested naming them *Helcosoma tropicum*. In 1901 W. Leishman found similar organisms in a smear from a spleen of a patient who died from "Dum-dum fever"; he published his observations three years later. In 1903 Ch. Donovan discovered similar parasites in spleens of kala-azar patients. These organisms were initially considered to be trypanosomes but Donovan described them as a new species, and in the same year Ross also linked them to kala-azar and suggested the name *Leishmania donovani*. Later Wright suggested the name *L. tropica* for the agent of cutaneous leishmaniasis [Rodjakin and Sukolin, 1999; World Health Organization, 2008].

Leishmania exist in two distinct forms: promastigotes, extracellular motile oblong organisms with a frontal flagellum that grow in insect gut and culture media, and amastigotes, intracellular roundish non-motile form with a short flagellum, in the mammal host. There are no reliable morphological differences between the promastigotes of different species. However, the amastigotes of some species may have morphological differences or characteristic distribution within host cells; for instance the amastigotes of *L. mexicana* are usually situated

peripherally in infected cells forming a characteristic "garland" [Adler and Gunders, 1964].

Historically, four overlapping periods of classification can be recognised, based on methods available during each period. In the first, the main criteria for classification were clinico-epidemiological and morphological. During that period the *Leishmania* of the Old World were initially classified as *L. donovani* that caused visceral leishmaniasis and *L. tropica*, the agent of localised cutaneous leishmaniasis. During the same period serological methods were applied to various strains to enable their differentiation. In the next period typing was based on iso-enzyme profiles (multilocus enzyme electrophoresis, MLEE). Most recently, PCR-based genetic methods were developed.

In 1913–1915 V.L. Yakimov, while studying leishmaniasis in Turkestan, discovered two morphological varieties of the parasite, *L. tropica major*, usually roundish, 5.5×4 μm, and *L. tropica minor*, usually oval, having the shape of rice grains, 4×3.25 μm. He did not link these varieties to distinct clinical forms. This was done in 1940s by P.V. Koževnikov, N.I. Latyshev and others at the Turkmen Dermato-Venerological Institute in Tashkent [Koževnikov, 1963]. Based on clinical and epidemiological data, they described two forms of the disease. The first was characterised as wet, with early necrosis, rural, and the other as dry, with late ulceration, urban. These were linked to the two varieties of *L. tropica*: *L. tropica major* and *L. tropica minor* respectively [Rodjakin and Sukolin, 1999], later promoted to species level, *L. major* and *L. tropica* based on clinical and epidemiological characteristics [Bray *et al.*, 1973; Bray, 1974]. In Balkh province, northern Afghanistan, where both forms co-exist, species identification for notification purposes is still performed based on size and number of intracellular parasites, using micrometer-fitted oculars [Faulde *et al.*, 2008] (these authors report different reference amastigote sizes: 1.5–2.5 μm for *L. tropica* and 4–5 μm for *L. major*; they also mention that the former usually

causes high burden of infection, up to 100 parasites per cell, while the latter less than 10, usually 2–4, amastigotes per cell).

The tendency to name the causative organisms of cutaneous leishmaniasis *L. tropica* persisted at least until the middle of the 20th century: in 1953 F. Biagi named the causative agent of localised cutaneous leishmaniasis in Central America "*L. tropica* var. *mexicana*" [Adler and Gunders, 1964].

Serological differentiation of the parasites was used starting from mid-1910s applying different techniques available at that time such as complement fixation, direct agglutination and lysis, which enabled grouping of the parasites into species or species complexes. Early experiments with direct agglutination showed that *L. donovani, L. infantum* and *L. canis* were serologically identical [da Fonseca, 1933]. Da Fonseca in 1933 used specific *Leishmania* anti-sera and was able to differentiate *L. braziliensis, L. tropica, L. donovani, L. infantum* and *L. canis*. Later on, tests using sets of monoclonal antibodies made it possible to differentiate serotypes within species [Schnur *et al.*, 2004].

Isoenzyme characterisation of *Leishmania* was developed during the 1970s, starting from single enzymes [Gardener *et al.*, 1974], then enzyme combinations [Chance *et al.*, 1977; Gardener, 1977]. To allow sufficient resolution multiple enzyme systems were employed [Kreutzer and Christensen, 1980].

Multilocus enzyme electrophoresis (MLEE) as applied at Université Montpellier is based on electrophoresis of 15 isoenzymes and has been performed there since 1981. It allows us to distinguish between species and, partially, to group strains at subspecies level. A group of strains displaying the same isoenzymatic phenotype is termed "zymodeme". Since 1989 a complementary technique, isoelectric focusing, is used [Université Montpellier 1]. It has higher resolution but is suitable for six enzymes only [Piarroux *et al.*, 1994]. MLEE remains the reference technique for *Leishmania* classification at species and sub-species level because during the last 25 years it has been

applied to the widest range and greatest number of strains compared to other methods [Schönian *et al.*, 2008].

At present the reference laboratory that maintains a cryobank of strains is the Laboratory of Parasitology and Mycology at Montpellier University. They have a searchable catalogue of reference strains on their site. According to isoenzyme profile a strain is assigned a MON-number. The numbers are assigned sequentially, so close numbers do not imply close isoenzymatic profiles. More than 3000 strains falling into more than 260 zymodemes are currently listed.

The catalogue lists seven strains of *L. tropica* isolated in 1990–1992 and 1997 in Syria (six from humans and one from a dog) that all belong to MON-76 group. Syrian *L. major* strains are MON-26, and *L. infantum* is MON-1. Manual search for *L. tropica* in neighbouring countries showed that they belong to other MON groups: the single isolate from Lebanon (dated 1994) was MON-6; four isolates from Iraq (three human isolates of 1965 and one from rat, of 1972) were MON-6, MON-7, MON-39 and MON-5; the only listed isolate from Israel, of 1996, is MON-137. Regrettably, no *L. tropica* isolates from Turkey are listed; those would be of special interest because leishmaniasis due to *L. tropica* is reported from the Turkish provinces of South-eastern Anatolia region and Hatay province, which are adjacent to the Syrian Aleppo Governorate. Overall, 38 strains of *L. tropica* are listed in the catalogue. They belong to 17 zymodemes. From some countries more than one zymodeme is listed but most zymodemes occur in one country only, with three exceptions: MON-6 was found in Iraq and Lebanon, MON-7 in Iraq, Jordan and Pakistan, and MON-137 in Egypt, Israel and Jordan. All MON-76 strains originate in Syria.

PCR-based molecular typing is based on amplification of nucleic acid fragments, then comparing them based either on their size or nucleotide sequences. Study of nucleotide polymorphisms began in the early 1970s, and phylogenies based on them largely confirmed the taxonomy based on

isoenzyme typing, with few exceptions. Genetic methods still suffer from some limitations, namely study of heterozygosity and recombination are not fully resolved for species that do not have haploid life stages [Schönian *et al.*, 2008].

Several methods are applied for characterisation of *Leishmania*. PCR-RFLP of cp8 and gp63, sequencing of ITS1 and multilocus sequence typing of housekeeping genes are suitable for species identification, whereas multilocus microsatellite fragment analysis and kinetoplast DNA typing are suitable for distinguishing single strains, the former can also be used to infer population structure [Schönian *et al.*, 2008].

A large study explored genetic relationship between 117 strains of *L. tropica* from multiple Asian and African locations, using 21 microsatellite loci [Schwenkenbecher *et al.*, 2006]. Turkish strains from all regions mostly belonged to one group, with two strains, from the South-East and Adana, forming a distinct group of their own. No strains from Syria were included in this study.

The results of these different methods of classification are broadly consistent in defining species complexes and, where applicable, the genetic relations between different species.

1.1.2. Transmission

Leishmaniasis is transmitted by the bite of an infected female phlebotomine sandflies. About 30 species are proven vectors. The *Phlebotomus* genus is divided into 12 subgenera and *Lutzomyia* into 25 subgenera and species groups [Killick-Kendrick, 1999].

The ability of a phlebotomine species to act as a vector depends on its co-existence with reservoir in the same geographic area, feeding preferences and susceptibility to infection. In susceptible phlebotomines the parasites attach to the epithelium of digestive tract (*Leishmania* subgenus to the midgut and *Viannia* subgenus, to hindgut), while in insensitive species the parasites would multiply while bloodmeal is being digested but are voided during

defaecation [Killick-Kendrick, 1999]. The structure of parasite lipophosphoglycan may play a role in the capacity of *Leishmania* to establish infection in specific vectors [Soares *et al.*, 2004]. However, the vectors themselves differ in their *Leishmania* specificity, some being more "permissive" than others, and the attachment of *Leishmania* to the epithelium in these "permissive" vectors is lipophosphoglycan-independent [Myšková *et al.*, 2007].

Historically, various insects were implicated in the transmission of leishmaniases, with more or less experimental evidence, such as fleas [Sergent *et al.*, 1912], lice and bed bugs [Wenyon, 1932], stable flies [Lainson and Southgate, 1965]. Moreover, while inducing leishmaniasis in laboratory animals or volunteers was possible through injecting them with either emulsion from sandflies or with promastigotes obtained from naturally infected sandflies, experimental transmission by a bite of a sandfly has been very difficult to achieve, which led to the suggestion of other modes of transmission, such as crushing or swallowing the insect [Adler, 1929]. However, in 1931 Shortt *et al.* succeeded in transmitting of *L. donovani* by the bite of *Ph. argentipes* to hamsters [reviewed by Sacks and Kamhawi, 2001], and later, in 1942, also by the bite of *Ph. argentipes* infected from a case of kala-azar, to five healthy volunteers [Swaminath *et al.*, 1942].

The sandfly seems to be the only plausible natural vector based on biological (development of the parasite in the gut), clinical (development of sores on exposed parts of the body where sandflies, unlike most other blood-sucking insects, bite) and epidemiological evidence (the distribution of cases follows the distribution of incriminated sandfly species) [Wenyon, 1932; Killick-Kendrick, 1999].

1.1.3. Visceral leishmaniasis

Visceral leishmaniasis has an estimated annual incidence of 500,000 cases with about 50,000 fatalities every year but these figures are likely to be an

underestimate. It is estimated that between 1984 and 1994 it caused 100,000 deaths in southern Sudan within a population of 280,000 [Chappuis *et al.*, 2007].

Zoonotic visceral leishmaniasis is caused by *L. infantum* in the Old World (Mediterranean region) and in the New World (Latin America), where the parasite is called *L. infantum/chagasi* [Desjeux, 2001; Berman, 2005]. Dogs and wild canids act as reservoir and *P. perniciosus, P. perfiliewi* and *P. neglectus* are among proven vectors [Killick-Kendrick, 1999].

Anthroponotic visceral leishmaniasis is caused by *L. donovani*. It occurs in the Indian subcontinent, south-east of Arabian Peninsula and East Africa causing severe epidemics [Desjeux, 2001]. *P. argentipes* is implicated in transmission in India and *P. orientalis* in Arabian Peninsula and East Africa [Killick-Kendrick, 1999].

1.1.4. Cutaneous leishmaniasis

The incidence of cutaneous leishmaniases (CL) is estimated at about a million cases a year, with 10 million people in 82 countries suffering from it. The annual incidence in the Middle East and the Maghrib is estimated at 350,000 cases [World Health Organization, 2007].

Because distribution areas of causative species overlap (Figure 1.1 [World Health Organization, 1997]), and parasites are not typed routinely, it is difficult to separately estimate the incidence and prevalence of zoonotic and anthroponotic forms in the Old World.

1.1.4.1. Zoonotic cutaneous leishmaniasis

Zoonotic cutaneous leishmaniasis is caused by *L. major* (Central and Western Asia and North Africa) and *L. aethiopica* in Ethiopia and Kenya [Reithinger *et al.*, 2007] in the Old World and by *L. (Leishmania) mexicana* and *L. (Viannia) braziliensis* complexes in the New World (Latin America). In the Old World the reservoir is small rodents (gerbils of genera *Psammomys, Meriones* and *Rhombomys* and the murine *Nesokia indica*) for *L. major* and rock hyrax (genera *Procavia* and *Heterohyrax*) for *L. aethiopica* [Bray *et al.*, 1973; Negera *et al.*,

2008]; and in Latin America, small rodents and big mammals of primary forest [Desjeux, 2001] such as sloths, porcupines and armadillos [Gentile *et al.*, 1981; Christensen *et al.*, 1982; Yadon *et al.*, 2003].

1.1.4.2. Anthroponotic cutaneous leishmaniasis

Anthroponotic cutaneous leishmaniasis (ACL) occurs in the Old World only, and is caused mostly by *L. tropica*, but also by *L. donovani* in India [Desjeux, 2001; Sharma *et al.*, 2005] It is mostly urban, reflected in old classification of leishmaniases into urban (dry) and rural (wet) forms. ACL is distributed mostly in Central Asia and Middle East, sometimes overlapping areas of zoonotic cutaneous leishmaniasis. In North Africa, the disputable species *L. killicki* causes chronic cutaneous leishmaniasis [Haouas *et al.*, 2005; Kallel *et al.*, 2005; Reithinger *et al.*, 2007; Harrat *et al.*, 2009].

The overall incidence is unknown but there are reports about increasing incidence of ACL in established foci such as Aleppo in Syria and Kabul in Afghanistan [Ashford, Kohestani *et al.*, 1992; Ashford *et al.*, 1993; Neouimine, 1996; Desjeux, 2001] and emergence of the disease in new foci in Algeria [Mihoubi *et al.*, 2008], Jordan [Nimri *et al.*, 2002], Morocco [Rhajaoui *et al.*, 2004] and Palestine [Al-Jawabreh *et al.*, 2004].

Man is considered to be the only reservoir of *L. tropica* [Desjeux, 2001; Minodier *et al.*, 2005]; but besides man, domestic and stray dogs [Dereure *et al.*, 1991] and rats of the genus *Rattus* [Neouimine, 1996] can become infected. In laboratory experiments, rats may infect sandflies even while remaining asymptomatic [Svobodová, Votýpka *et al.*, 2003]. Although no definite evidence exists yet that any species other than man can serve as disease reservoir, this possibility should not be excluded [Al-Jawabreh *et al.*, 2004], rock hyrax being one of suggested candidates based on epidemiological association [Jaffe *et al.*, 2004].

ACL is transmitted by *Phlebotomus sergenti*. Sandflies breed in warm, humid environments with decomposing organic matter, such as piles of waste, open

sewage ducts [Desjeux, 2001], and in sheds and cellars [Svobodová, Sádlová *et al.*, 2003]. They are weak fliers, so they rest and feed within a few hundred metres of their breeding places [World Health Organization, 1997]. Besides humans, they feed on other warm-blooded animals, such as goat, sheep, chicken, rat and house mouse [Svobodová, Sádlová *et al.*, 2003]. Biting activity starts after sunset and continues until dawn. During daytime they rest in dark humid places: sheds, rooms, caves, crevices [World Health Organization, 1997]. Thus, the most important vector control activities are environmental sanitation [Mott *et al.*, 1990], residual indoor insecticide spraying and using ITNs [Davies *et al.*, 2003].

1.2. Leishmaniases in Syria

1.2.1. Geography

Syria is a country in the Eastern Mediterranean region. It is divided to 14 major administrative divisions called governorates. Aleppo is the centre of Aleppo governorate and is the second largest city (after the capital Damascus), located in the north (36° 12' N, 37° 10' E), bordering Turkey (Figure 1.2). Population estimate for the city is 1,671,673 (2008) [Wikipedia, 2008] and for the governorate, 4,281,000 (2006) [Central Bureau of Statistics, 2007].

1.2.2. History

Cutaneous leishmaniasis has been known in Aleppo since ancient times, giving the disease one of its names, Aleppo boil. "One of the first and most important clinical descriptions was made in 1756 by Alexander Russell following an examination of a Turkish patient" [World Health Organization, 2005]. He mentioned the local name for the disease, *habt as-sineh*, which he translated as "botch of a year", and attributed it to contaminated water sources [Russell, 1756].

1.2.3. Distribution

In Syria, both anthroponotic and zoonotic (ZCL) forms of leishmaniasis occur, of which ACL is responsible for 65%—93% of reported cases (estimated by adding up numbers from Aleppo and four adjacent governorates believed to be affected by ACL, Figure 1.3 and Figure 1.4). In addition, there are rare cases of zoonotic visceral leishmaniasis (ZVL) due to *L. infantum* (tens of cases reported yearly) [Abazid, 2000], *see* Table 1.2.

ZCL, due to *L. major*, occurs in semi-desert areas to the east of Damascus and in north-eastern governorates, mainly Hasaka and Deir az-Zawr, where several rodent species (*Psammomys obesus, Meriones crassus, Nesokia indica*) serve as reservoirs, and *Phlebotomus papatasi* is incriminated as the vector. ZVL, with a canine reservoir, is confined to several foci in the coastal region (Lattakia and Tartous), the north (Aleppo and Idlib) and the south, near the border with Jordan [Al-Nahhas *et al.*, 2003]. *Ph. tobbi* is the implicated vector [Intersectoral Committee for Vector Control, 2006]. ACL occurs in the Northern and Coastal regions. The putative vector is *Ph. sergenti* [Jalouk *et al.*, 2007].

In 1980s MoH established a reporting system for leishmaniasis with few thousand cases reported every year. During 1990s the annual recorded incidence was over 11,000 in all but one year (in 1998 it was 8893 cases). During 2000—2005 the annual recorded incidence was in the range 19,837—29,100, then, in 2006 and 2007 decreased to 18,732 and 17,709 respectively [al-Shammas, 2008]. This can be partly attributed to improvement in reporting, expansion of diagnosis and treatment services and active case detection in schools and villages but a real increase is possible. It was estimated [Ashford *et al.*, 1993] that in early 1990s the passive case detection system in Aleppo recorded and treated only 1 patient in 4. There have been no more recent estimates of detection rate.

1.2.4. Structure of relevant health services

Syria is divided administratively into 14 governorates. Central executive power is represented by the Council of Ministers.

Medical practice in the Syrian Arab Republic is regulated by the Ministry of Health (MoH) in cooperation with relevant professional associations. MoH issues practice licenses to physicians, dentists, pharmacists and nursing staff.

Medical services are provided through public and private health care facilities, in addition to charities.

Public sector health care facilities belong to one of four ministries: Health, Higher Education, Defence or Interior. The former two offer health care to the general public, while the latter two cover their staff and staff's family members.

Public health care facilities of MoH include primary health care (PHC) centres, specialised polyclinics, secondary-level hospitals in every governorate (one or more) and specialised tertiary hospitals in large cities, such as Damascus and Aleppo. There used to be no strict referral system: for example, anyone could come to outpatient clinics in a tertiary hospital and be admitted if deemed necessary provided free beds were available, although health district policy did exist on paper. Starting from 2007, health district policy was enforced. Every family was required to be registered in a PHC centre of their choice. This centre would be their first point of encounter with the health system. All referrals to specialised clinics or hospitals, except emergencies, can only be issued by the PHC centre where the family is registered.

Ministry of Higher Education runs only tertiary university hospitals in the three cities where universities have faculties of medicine (Damascus, Aleppo and Lattakia; recently, a faculty of medicine was established in al-Baath University in Homs). These hospitals have outpatient departments and emergency departments. There is no formal referral system, and these hospitals serve citizens regardless of their place of residence.

Public health care facilities currently remain mostly free of charge with few exceptions. Free services include diagnosis, treatment, and, for in-patients, also bed and three daily meals. If a diagnostic procedure or a drug is not available in a PHC centre or a hospital, the patient refers to private-sector laboratories or pharmacies.

Private sector facilities are either small private clinics or, rarely, small specialised centres operated by a few physicians with specific area of specialist service, in addition to private hospitals which are usually small. Virtually all pharmacies are private, as are independent laboratories (those outside health care facilities). Fees are usually paid out-of-pocket.

Charities run small polyclinics with several physicians — general practitioners and sometimes part-time specialists, free of charge or charging a symbolic fee. They may be equipped with a small lab with basic tests.

Only health care facilities run by the MoH are obliged to report leishmaniasis cases. Other facilities may or may not do so.

Import of antimonials is monopolised by the MoH, which distributes them to its own facilities and to Ministry of Defence, but not to pharmacies. However, drugs may be smuggled in from neighbouring countries, or, probably, leak from public facilities, although I am not aware of any cases of the latter.

Pentostam® and Glucantime® are the two drugs used in MoH system. Glucantime (Sanofi-Aventis, France) is the first-line drug for intralesional treatment; Pentostam (GlaxoSmithKline, UK) is used either intralesionally, if Glucantime fails, or intramuscularly, and its use is normally restricted to Leishmaniasis Control Center, but may be distributed to peripheral health centres with directions to use it for intralesional treatment instead of Glucantime when large stocks with expiry date in the next six to nine months exist. Pentostam is deemed more efficient but also more painful when given intralesionally. Several years ago MoH imported generic SSG from India (Albert David, Kolkata) but received unfavourable reports about its efficiency from the

governorates and returned to importing the brand products [Dr. Lama Jalouk, personal communication].

MoH has two kinds of divisions, both called "directorates": *specialised*, that run their programmes on the country level (e.g. Directorate of Chronic and Endemic Diseases responsible, among other things, for leishmaniasis surveillance and control programme, etc.) and *geographical*, by governorate (e.g. Aleppo Health Directorate, etc.), which are responsible for running all MoH health services and programmes on the governorate level.

In most governorates, the Directorate for Chronic and Endemic Diseases is represented by a specialised "Malaria, Leishmaniasis and Schistosomiasis Control Centre". In Aleppo *Leishmaniasis Control Center* (LCC) used to be independent due to the importance of the disease in that governorate.

Malaria currently occurs in Syria sporadically, and most cases are said to be imported, so it does not constitute a major public health problem. The import and distribution of antimalarial drugs are also controlled by MoH. The main role of antimalarial clinics is to dispense chloroquine based on prescriptions from government-run clinics, mostly for its anti-inflammatory properties in chronic autoimmune conditions. A similar policy applies to other "dual-use" drugs, such as those used in the treatment of tuberculosis.

1.2.5. Control programme

Syria has had a surveillance system for malaria since 1950s, which aimed at eradicating malaria in accordance with World Health Organization (WHO) programme. In 1986 surveillance of leishmaniasis was added to this programme. Monthly lists with the names of the patients referring to specialised centres were compiled and sent to MoH. In 1994 special forms for data collection were introduced; these included patient forms, monthly case report forms and daily and monthly vector surveillance forms. Patient and monthly report forms included basic epidemiological information. These forms have been used since then until the present.

Forms and registries for routine case data collection and reporting, and how they are used, are described in more detail in Chapter 2.

The leishmaniasis control programme in Syria aims to decrease disease incidence in endemic areas and to continue surveillance and control activities in old and emergent foci by breaking transmission cycle between vector and human host [Abazid, 2000].

Specialised control centres in governorates implement the control programme. It consists of early diagnosis and treatment to self-referring leishmaniasis patients, surveys in schools, especially primary schools with pupils of ages 6-13 but also in summer student camps (in collaboration with Directorate for School Health of Ministry of Education), surveys in newly affected villages or areas with sharp increase in incidence, vector surveillance in selected houses using overnight paper sheets with castor oil and two campaigns a year of residual insecticide spraying in villages and city districts with incidence rate greater than 1% in the previous year. For ZCL, deep ploughing of rodent-infested areas may be performed in cooperation with Ministry of Agriculture.

In the army, control measures are performed by the Ministry of Defence (MoD) Medical Services Administration. Insecticide spraying, ploughing, case detection and treatment are among the activities performed. Reports of the activities and case statistics are usually inaccessible. One anecdote reports that once the military performed deep ploughing and eradication of chenopods (a species of *Atriplex*, most probably, the Mediterranean salt-bush *Atriplex halimus*), known to be the preferred food for the fat sand rat *Psammomys obesus* [Aharonson *et al.*, 1969], the main animal reservoir of ZCL in the area of Dmair near Damascus [Khiami *et al.*, 1991]. In the next few years MoH noted the sharp drop in CL incidence in the area and had difficulty explaining it because they had no idea about MoD activities in that area.

It seems that above-mentioned measures generally fail in reducing the incidence of CL in Syria [Jalouk *et al.*, 2007]. Possible explanations include the retroactive nature of spraying campaigns which are based on previous year incidence, possible inadequacy in spraying plan, if potential sandfly breeding sites are missed, inadequate case detection leaving a sufficient reservoir for further spread of infection, etc.

1.3. The disease

1.3.1. Clinical classification

The eco-epidemiologic classification presented above is deliberately simplified to serve its purpose. The clinical course of leishmaniases differs strikingly depending on causative species and host characteristics. Clinically, leishmaniasis is classified into two, visceral (VL) and tegumentary [e.g. Azeredo-Coutinho *et al.*, 2007], or three broad categories, where the latter form is divided into cutaneous (CL) and mucocutaneous (MCL) leishmaniasis [e.g. Herwaldt, 1999]. The visceral form includes viscerotropic forms.

Cutaneous forms are further subdivided into localised (LCL), disseminated (or diffuse, DCL), *leishmaniasis recidivans* (or *recidiva*), post-kala-azar dermal leishmaniasis (PKDL) [Kenner and Weina, 2007] and chronic (CCL) [Kallel *et al.*, 2005]. Some authors [Herwaldt, 1999] do not consider chronic and recidivant leishmaniasis different forms of the disease.

Clinical manifestations of infection depend on host characteristics (nutritional and immune status), parasite species and virulence, localisation, complications and other factors [Rodjakin and Sukolin, 1999].

1.3.1.1. Visceral leishmaniasis

Visceral leishmaniasis is the most severe form of the disease. Overt disease is almost universally fatal if left untreated [Herwaldt, 1999; Lira *et al.*, 1999]. It manifests by fever (local name for the disease is *kala-azar*, i.e. "black fever" in Hindi), weight loss and enlargement of spleen and liver.

Differences in disease epidemiology between the Indian and Mediterranean forms were noted before it was known that they are caused by different species of *Leishmania*: in Mediterranean, or infantile, VL most cases (about 90%) in endemic areas are less than five years old, whereas in India this age group was affected in only 4–30% of cases; in addition, the incidence rate of VL in Italy is remarkably lower than in India. Absence of infection in dogs in India, despite large numbers examined, was also noted [Adler, 1929]. However, a recent paper from Sri Lanka reported isolation of *L. donovani* from two of 151 pet dogs examined in foci of cutaneous leishmaniasis caused by the same parasite. The authors considered the available evidence to be insufficient to incriminate dogs as reservoir and emphasised the need for further studies [Nawaratna *et al.*, 2009].

L. donovani in the Indian subcontinent, south-east of Arabian Peninsula and East Africa is believed to be anthroponotic and may cause severe epidemics. For example, MSF Holland reported 100,000 VL deaths in southern Sudan in early 1990s in a population smaller than one million [Desjeux, 2004].

Visceral leishmaniasis caused by *L. infantum* is zoonotic. It has similar clinical manifestations but has a different age group preference. Numbers of disease cases are relatively low among inhabitants of endemic areas, and many instances of infection remain asymptomatic. In a study in two foci in southern Syria where a seroprevalence survey using rK-39 was performed [Al-Nahhas *et al.*, 2003] and all children between 6 months and 6 years of age were screened, about a quarter (80/345) were seropositive, and of these only ten cases (1 in 8) were symptomatic (irregular fever, weight loss and mild, 2–3 cm, hepatosplenomegaly). After nine-month follow-up most positive asymptomatic children seroconverted to negative, and in four of those who remained positive only lymphadenopathy was detected. Bone marrow aspirates were obtained from ten asymptomatic cases and amastigotes were detected in all of them, although in low numbers compared to symptomatic patients.

There are also reports of *L. tropica* causing "visceralising" disease in immunocompetent persons. In a small case series [Magill *et al.*, 1993] the disease had non-specific manifestations and was not usually accompanied by organomegaly, fever or anaemia.

1.3.1.2. Cutaneous leishmaniasis

1.3.1.2.1. Localised cutaneous leishmaniasis

Localised cutaneous leishmaniasis manifests with one or more lesions, usually on uncovered body parts. It is mostly associated with *L. tropica, L. major* and *L. aethiopica* in the Old World but *L. donovani* and *L. infantum* can also cause localised cutaneous disease; in fact, most of cutaneous leishmaniasis in France and Spain, previously attributed to *L. tropica*, is caused by *L. infantum*. In recent papers cutaneous leishmaniasis due to *L. donovani* was reported from Sri Lanka which was also found to infect dogs [Nawaratna *et al.*, 2009], whereas in India this parasite could not be isolated from dogs [Adler, 1929]. So, it seems that all Old World species can produce cutaneous forms. In the New World all the species, except *L. infantum*, produce mostly LCL.

Leishmania major infections tend to have a shorter course compared to *L. tropica*, with shorter incubation period, larger and more inflamed lesions, earlier ulceration and epithelialisation [Koževnikov, 1963; Rodjakin and Sukolin, 1999].

1.3.1.2.2. Disseminated cutaneous leishmaniasis

Disseminated cutaneous leishmaniasis is caused by *L. aethiopica* in the Old World and *L. amazonensis* and *L. mexicana* in the New World [Reithinger *et al.*, 2007].

1.3.1.2.3. Leishmaniasis recidivans

Follows a healed *L. tropica* infection which usually manifests as small non-inflamed satellite lesions that appear around the scar.

1.3.1.2.4. Post-kala-azar dermal leishmaniasis

Post-kala-azar dermal leishmaniasis is a complication of kala-azar caused by *L. donovani* in India and Sudan and follows successfully treated VL. In India about 50% of cases suffer from the condition, which typically starts 2-3 years post-cure and requires prolonged treatment; in Sudan it affects 5-10% of cases, starts 0-6 months post-cure and tends to resolve spontaneously, and only severe cases are treated [Zijlstra *et al.*, 2003].

1.3.1.2.5. Chronic cutaneous leishmaniasis

Chronic cutaneous leishmaniasis is usually a complication of ACL caused by *L. tropica*, when a lesion fails to heal within 12-18 months, with or without treatment, and satellite lesions appear at its border. Chronic CL is also reported from Tunisia as a separate entity where it is caused by *L. killicki* [Haouas *et al.*, 2005; Kallel *et al.*, 2005]. *Leishmania killicki* was characterised as a separate species by isoenzyme typing but molecular analyses classify it as *L. tropica* [Schönian *et al.*, 2008].

ZCL caused by *L. major* usually heals in few months, even without treatment, and was previously believed not to cause chronic lesions. However, wide application of leishmanisation during the Iran–Iraq war permitted detection of a few cases that failed to heal normally and remained active for several years despite treatment.

1.3.1.3. Mucocutaneous leishmaniasis

Mucocutaneous disease is caused by certain New-World species (Table 1.1) and is associated with destruction of nasal, oral and pharyngeal mucosa resulting in severe mutilation [Desjeux, 2004], social stigma [World Health Organization, 2008] and is potentially fatal [Stark, 2008]. It may appear after the cutaneous disease has healed or present without evidence of prior cutaneous disease.

Rare cases of involvement of mucous membranes was reported by one group from the Old World (Tunisia), due to *L. major* [e.g. Kharfi *et al.*, 2003;

Kharfi *et al.*, 2004]. They noted relatively benign course with no subsequent mutilation. However, the lesions were mostly located on the lips or nose, with only one case having clearly intra-nasal localisation.

1.3.1.4. Leishmania/HIV co-infection

Co-infection with human immunodeficiency virus (HIV) is an additional challenge to leishmaniasis control. HIV infection and other immunosuppressive states increase the risk for the patients to develop a visceral disease. The main risk group in Western Europe is intravenous drug users [Desjeux, 2001]. Up to 70% of VL cases in Southern Europe are associated with HIV. Overlapping areas of HIV and leishmaniasis lead to changes in epidemiology due to disease activation in asymptomatic carriers and spread of VL to cities with a potential to cause epidemics. Co-infection was reported from 34 countries so far (Figure 1.5) [World Health Organization, 2008].

1.3.2. Pathogenesis

When an infected female sandfly takes a bloodmeal, it inoculates promastigotes into the skin; the typical number of injected parasites is 10^2-10^3, and these are phagocytised by leucocytes. Then the promastigotes undergo morphological and metabolic modifications within the macrophages. They transform to amastigotes which have a roundish shape with short flagellum [Roberts, 2006].

The first cells that arrive at the site of infection are polymorphonuclear neutrophils. Within them the promastigotes do not change nor multiply. Then they can use the apoptotic neutrophils to silently enter their final host, the macrophage, where they convert to the amastigote stage [van Zandbergen *et al.*, 2006].

It was noted that fresh promastigote cultures are less infective than older cultures that contain short metacyclic and non-flagellate forms [Berberian, 1939; Row, 1939]. In recent studies it was shown that the inoculum must contain some apoptotic promastigotes which display the apoptotic "eat me" signal,

silencing the initial immune response and allowing viable *Leishmania* to evade the defence of the host. Apoptotic promastigotes displayed phosphatidylserine on their membrane, and this compound is known to silence phagocyte functions and is associated with secretion of transforming growth factor β (TGF-β) and interleukin (IL) 10, and suppression of tumour necrosis factor α (TNF-α), so apoptotic cells do not provoke an immune response [van Zandbergen *et al.*, 2006].

Amastigotes are enclosed in so-called parasitiphorous vacuoles, which form from original phagosome after fusion with lysosomes and endosomes. They endure the acidic environment of these vacuoles and are able to feed on vacuole contents. Parasites replicate slowly, each replication cycle taking about 24 hours. [Decuypere, 2007].

1.3.3. Role of sandfly inoculum

Sandfly saliva possesses vasodilatory, immunomodulatory and anticoagulant properties which may contribute to evasion of host immune response by the parasites and was shown in animal studies to exacerbate *Leishmania* infection [Giunchetti *et al.*, 2008]. Sandfly bites evoke a delayed inflammatory response, and the period between the bite and the response shortens with repeated exposure [Theodor, 1935]. It was shown that mice vaccinated with maxadilan or 15 kDa salivary protein became unresponsive to infection with *L. major* when injected with promastigotes and saliva [Giunchetti *et al.*, 2008], making it a candidate for vaccine development. Another study demonstrated that repeated sandfly bites provoked cellular and humoral immune response in mice, and the response was specific for the sandfly species (*Ph. sergenti*). Cross-reaction with the salivary gland homogenates of *Ph. papatasi* was partial, and there was no cross-reaction with *Ph. arabicus* [Drahota *et al.*, 2009].

A series of studies demonstrated the role of promastigote secretory gel (PSG) in the development of leishmaniasis. Promastigote secretory gel is a gel-

like substance produced by the promastigotes that blocks the anterior midgut of the sandfly [Rogers *et al.*, 2002], and its main component is filamentous proteophosphoglycan (fPPG) which is injected during the bloodmeal with the promastigotes and has a main role in exacerbating the infection [Rogers *et al.*, 2004] by enhancing early recruitment of macrophages to the site of infection and upregulating the macrophage arginase activity [Rogers *et al.*, 2009]. In addition, the PSG plug modifies the behaviour of the sandfly making it more likely to return to feeding if interrupted and bite more hosts [Rogers and Bates, 2007].

1.3.4. Immunology (in humans)

Old-World CL usually cures spontaneously and is believed to confer life-long immunity [Scott *et al.*, 2004; World Health Organization, 2005].

Publications about immune response to *L. tropica* in humans are extremely rare; most research addresses *L. major*, *L. donovani* and Latin American species.

In general terms, the course of the disease is determined by the interaction between the parasite species and the host response. The immune response ranges from hyperergic reactions with low parasite burden, prominent local tissue destruction and strong cell-mediated immunity and delayed-type hypersensitivity, exemplified by the chronic cutaneous and mucocutaneous disease, to asymptomatic disease, self-healing localised or oligosymptomatic visceral infections, to near-complete anergy with high parasite burden, as in diffuse cutaneous leishmaniasis, manifest visceral leishmaniasis and post-kala-azar dermal leishmaniasis [Murray *et al.*, 2006]. The species usually associated with visceral leishmaniasis may also cause cutaneous forms of the disease. Thus, *L. donovani* was reported to cause CL in India [Sharma *et al.*, 2005] and Sri Lanka [Siriwardana *et al.*, 2007; Karunaweera, 2009], most CL cases in Southern Europe are caused by *L. infantum*, and human cutaneous cases are more numerous than visceral ones [Pratlong *et al.*, 2004]. On the other hand, several cases of *L. tropica* causing oligosymptomatic visceral disease in apparently

immunocompetent adults were reported [Magill *et al.*, 1993] and one case from Iran of classical infantile VL from which *L. tropica* was isolated [Alborzi *et al.*, 2006].

A longitudinal study in a focus of *L. infantum* infection in Iran performed using leishmanin skin testing and direct agglutination test to measure the dynamics of cellular and humoral immunity [Davies and Mazloumi Gavgani, 1999] suggested that humans may acquire protection against infection with age which is independent of exposure to the parasite, probably due to immunogenic properties of sandfly saliva.

In a study of patients with CL due to *L. major* in Iran, cure was associated with proliferation of interferon-γ (IFN-γ) producing CD4+ cells, while in patients with non-healing lesions low levels of IFN-γ and high levels of interleukin-4 were detected [Ajdary *et al.*, 2000].

1.4. Clinical course of ACL

After a long incubation period which usually lasts 3-6 months but may extend for more than a year, a small papule appears on the location of parasite inoculation, usually on an uncovered part of the body, which slowly enlarges and reaches 3-6 mm, sometimes up to 20 mm during the course of 3-4 months, and becomes a red brownish colour. A characteristic central depression may develop, which disappears when scaling starts. Exudate may appear and form a crust. The crust falls out exposing an ulcer. The papule usually ulcerates 4-8 months after appearing but ulceration may be delayed for longer than one year. After ulceration it takes about 3 months until epithelialisation starts. Epithelialisation is usually complete in 1-2 months, leaving a depressed scar. The period from primary papule to scar formation varies from 6 months to 2 years but is usually between 6 months and one year [Rodjakin and Sukolin, 1999; World Health Organization, 2005].

Two atypical forms are described: (a) chronic (lupoid), usually defined as persistence of the lesion for >1.5–2 years, frequently despite treatment; and

(b) recurrent leishmaniasis, when new elements appear on or around a healed scar, usually within 2 years [Marovich *et al.*, 2001]. The former constituted 3%, and the latter ~6% of patients in Turkey [Gürel *et al.*, 2002], while in Aleppo the lupoid form was reported in >30% [Douba *et al.*, 1997], but the study was based in a specialised dermatology referral centre. Some authors [Herwaldt, 1999] do not make a distinction between these two entities, which may be justified because they cannot be reliably differentiated based on histopathological findings, and the clinical history is not always reliable [Douba *et al.*, 1997].

Secondary infection may complicate the course of ACL, and often responds to topical antibiotics [Alrajhi *et al.*, 2002].

Table 1.3 shows a classical comparison between ZCL and ACL [Rodjakin and Sukolin, 1999].

1.5. Diagnosis

Primary diagnosis is based on clinical history, characteristic appearance of the lesion (for CL) or the systemic signs and symptoms (for VL) upon examination, and the epidemiological situation. The diagnosis is usually confirmed by visualising the parasites in pathological material.

1.5.1. Diagnosis of visceral leishmaniasis

The diagnosis of VL is usually confirmed by microscopical examination of spleen or bone marrow aspirates. In addition, the pathological material can be cultured to detect promastigotes, and several serological tests were developed, such as ELISA, fluorescent antibodies direct agglutination test (DAT) and rK39 (a rapid dipstick test based on detection of serum IgG antibodies [Singh *et al.*, 2009] to recombinant K39 antigen based on 39 amino-acid repeat of *L. chagasi* [Chappuis *et al.*, 2005]). The serological tests may give false-positive results in patients who have recovered or had asymptomatic disease [Davies and Mazloumi Gavgani, 1999; Al-Nahhas *et al.*, 2003; Terán-Angel *et al.*, 2007]. However, in a recent report it was shown that rK39 test performed on sputum

has higher specificity, although marginally lower sensitivity, than the same test applied to serum [Singh *et al.*, 2009].

1.5.2. Diagnosis of cutaneous leishmaniasis

The diagnosis of cutaneous leishmaniasis is confirmed by demonstration of parasites in pathological material from lesions. This can be done by making slit-skin smears, punch biopsies or lesion scrapings. Quantity of the parasites in the lesions tends to decrease with increasing age of the lesion, [Borovsky, 1898; Herwaldt, 1999] making it harder to obtain parasitological confirmation. In Aleppo, positivity rates achieved using smears of material obtained from lesion scraping using a lancet blade are between 70-80% for lesion durations of 1-9 months, with best results in lesions of 2-4 month duration. Giemsa-stained smears may take as little as 15 minutes to provide a result if done by experienced dedicated laboratory staff. Smears are examined microscopically at 1000x magnification.

Culture is less sensitive than microscopy [Alrajhi *et al.*, 2002], more expensive and time-consuming, and is not routinely used.

PCR-based diagnosis is promising, offering high sensitivity and specificity but is still expensive and sophisticated, and not applied routinely. Leishmanin skin testing may be used for screening purposes but not for confirming active disease, at least in endemic areas. Serodiagnostic techniques are also of limited value in detecting active disease.

1.6. Treatment

1.6.1. Antimonial compounds

Pentavalent antimonials (Sb^V) are considered the first-line treatment of leishmaniases. Two preparations are available commercially, sodium stibogluconate (SSG; e.g., Pentostam) and meglumine antimoniate (MGA; e.g., Glucantime). Neither of them is superior; their efficacy depends on antimony content (100 mg/ml in Pentostam and 81 mg/ml in Glucantime). The exact

mechanism of action of antimonials is only now becoming clear [Croft *et al.*, 2006]. Treatment is given either locally (intralesional infiltration, i.l.) or systemically. Systemic SSG can be given intramuscularly (i.m.) or intravenously (i.v.), while MGA intramuscularly only [Blum *et al.*, 2004], in doses of 10-20 mg Sb^V/kg/day for 20 days. Antimonial preparations are toxic, costly, and intramuscular and intralesional administration is very painful.

In the Old World, intralesional therapy (infiltration of upper- and mid-dermis from multiple sides until the lesion is completely blanched [Uzun *et al.*, 2004]) has the advantage of low systemic dose, hence less side effects, but cannot be applied when lesions are numerous, large or in a difficult location, e.g. ears or eyelids. In these cases, or when intralesional treatment fails, systemic therapy is given. Reported intralesional treatment regimens vary from daily [Tallab *et al.*, 1996] or every other day [Tallab *et al.*, 1996; Alkhawajah *et al.*, 1997], twice a week [Chiheb *et al.*, 1999; Salmanpour *et al.*, 2001], weekly [Mujtaba and Khalid, 1999; Asilian *et al.*, 2003], fortnightly or three injections every other day repeated every month until cure [Sharma *et al.*, 2005]; the treatment course varies between three [Tallab *et al.*, 1996; Douba *et al.*, 1997; Asilian *et al.*, 2003] and up to 20 injections [Uzun *et al.*, 2004]; in Aleppo, Syria, up to 24 injections with weekly intervals are given. Treatment may continue either for a fixed number of injections or to a certain maximum unless the lesions cure earlier.

1.6.2. Other pharmacological agents; immune therapy

A number of other treatments have been investigated, both systemic (injectable or oral) and local, given either alone or in combination with antimonials. The response to the treatment varies according to the species, so investigation of new drugs should be "species-specific"; yet frequently treatment studies are performed without defining the species. Clinical trials of *L. tropica* are rare: one in Syria [Harms *et al.*, 1991], one in Turkey [Özgoztaşı

and Baydar, 1997], and one in Afghanistan [Reithinger, Mohsen *et al.*, 2005] were identified.

Examples of systemic treatment are [Blum *et al.*, 2004] imidazoles, such as itraconazole [Dogra and Saxena, 1996] and fluconazole [Alrajhi *et al.*, 2002]; amphotericin B (standard or lipid formulations) [Alvar *et al.*, 2006]; miltefosine [Soto and Berman, 2006]; or immunotherapy [Convit *et al.*, 2003].

As local treatment, different formulations of paromomycin ointment were found moderately effective in both forms of Old World CL [El-Safi *et al.*, 1990; Ozgoztasi and Baydar, 1997], and a new topical formulation, WR279,396, a hydrophilic ointment with 15% paromomycin and 0.5% gentamicin was tested against *L. major* CL in Tunisia and France with good effect but also with surprisingly high effectiveness of the placebo [Ben Salah *et al.*, 2009]. Immunomodulation with topical imiquimod was tried without decisive advantage [Arevalo *et al.*, 2001; Firooz *et al.*, 2006]. A single patient report mentioned successful treatment of *L. major* CL in an infant using topical colloidal solution of amphotericin B [Zvulunov *et al.*, 2003]. Intralesional metronidazole was proposed by one group [Al-Waiz *et al.*, 2004]

All the treatments mentioned are either toxic (e.g. amphotericin B; lipid formulations are less toxic but more expensive) or their efficiency is low or not confirmed in larger well-designed trials; many of the conducted trials, even those with randomised controlled design, are of low quality, with small numbers of patients, inconsistent methods and end-points [Khatami *et al.*, 2007]. The same conclusion is supported in a recent Cochrane review of 49 randomised controlled trials of CL. For *L. tropica*, the authors made three positive statements: there was reasonable evidence that itraconazole 200 mg/day is better than placebo, intralesional SSG is better than intramuscular SSG, and thermotherapy is better than intramuscular SSG [González *et al.*, 2008]. There is an urgent need for new acceptable treatments for all forms of leishmaniasis [Alvar *et al.*, 2006; Modabber *et al.*, 2007].

Antimonial compounds, given either intralesionally or parenterally, are the first line of treatment in most settings. Pentamidine is used in French Guyana, especially for CL caused by *L. guyanensis* because it is unresponsive to antimonials [Minodier and Parola, 2007]. A different approach was recommended by Soviet authors [Shuvalova, 2001]: in fresh lesions she recommends quinacrine, monomycin (paromomycin), berberine sulphate or methenamine, either intralesionally or as ointments or lotions. In advanced cases, systemic monomycin or quinacrine is given. Antimonials are reserved for severe cases.

1.6.3. Physical treatments

Surgical excision, cauterization, cryotherapy and local heat are applied. For cryotherapy, liquid nitrogen or dry ice is used. Heat is applied directly (from infra-red source) [Rahim and Tatar, 1966] or generated by radio-frequency device [Reithinger, Mohsen *et al.*, 2005]. Uncontrolled studies show near 100% effectiveness. Controlled studies are rare, and tend to produce less impressive results, of effectiveness roughly similar or a little inferior to that of intralesional antimonials [Blum *et al.*, 2004]. A few reports on use of ablation with CO_2 laser were located, most of them published in 1985-1993, all from USSR, except one from Cuba, and a more recent one from Iran [Asilian *et al.*, 2004]. The results seem encouraging: for example, out of more than 100 patients treated in the USSR in 1980s, no relapses were noted among 82 (76%) patients who could be followed up after 7 years [Babajev *et al.*, 1991]. In one Iranian study laser treatment was more effective than Glucantime, had less side effects and shorter time to cure (1 vs. 3 months) [Asilian *et al.*, 2004].

Several studies from Iraq reported very high efficacy of several treatment modalities, such as local heating, intralesional hypertonic (7%) saline, intralesional [Sharquie *et al.*, 1997] or oral [Najim *et al.*, 1988; Sharquie *et al.*, 2001] zinc sulphate, direct current [Sharquie *et al.*, 1998]; but they were either

not confirmed in independent studies or the results were unimpressive [e.g. Firooz *et al.*, 2005].

1.7. Antimonial tolerance

All the papers describing treatment of CL report varying proportion of treatment failures. Although the results cannot be compared due to differences in study settings, designs and end-points, it can be stated that no single treatment is proven to be highly and uniformly effective.

Possible reasons for treatment failures are parasite factors, such as virulence and drug resistance, host factors, such as immunity and nutritional status, poor compliance and errors in administration of therapy [Douba *et al.*, 1997; Rodjakin and Sukolin, 1999]. Thus, it is desirable to differentiate between drug resistance and treatment failure in general, and define the former as increased tolerance of the parasite to the studied drug, confirmed using appropriate laboratory techniques.

Most treatment studies concentrate on comparing the effectiveness of different treatment options. Only in some publications are failures discussed, and even less publications explore the association between clinical treatment failures and parasite susceptibility. Most such papers explore the more severe forms of leishmaniases, i.e., visceral and mucocutaneous disease; ACL remains largely unexplored.

Determining *in vitro* resistance requires using a dividing population of the parasite stage found in mammal host [Croft and Brun, 2003], in case of *Leishmania*, amastigotes. A study of treatment failures in kala-azar in India [Lira *et al.*, 1999] found a strong correlation between clinical response and *in vitro* sensitivity of *L. donovani* to sodium stibogluconate in amastigote, but not promastigote assays. However, Azeredo-Coutinho *et al.* reported a correlation between clinical response and sensitivity to MGA of both amastigote and promastigote stages of *L. braziliensis*, and a positive correlation between fifty-percent effective concentration (EC_{50}) values of both stages, although the *in vitro*

sensitivity of promastigotes was about 100 times lower compared to amastigotes [Azeredo-Coutinho *et al.*, 2007]. Another study of American tegumentary leishmaniasis caused by three species found correlation between pre-treatment antimony sensitivity level of intracellular amastigotes and clinical outcome, and that sensitivity of the parasites isolated from treatment failures was less than pre-treatment strains [Rojas *et al.*, 2006].

Culturing of amastigotes requires application of tissue culture techniques, such as macrophage-amastigote system, because in usual culture media *Leishmania* transforms to promastigotes, that are far more resistant to antimonials *in vitro* [Croft, 1986]. Until now, only one study assessed drug sensitivity of *L. tropica* and linked it to clinical outcomes [Hadighi *et al.*, 2006].

The precise mechanism of action of pentavalent antimonials is not fully clarified. It is agreed that Sb^V is reduced to trivalent antimony, which is the active form of the drug, in the macrophages or in the parasites [Croft *et al.*, 2006]. Most studies were performed on laboratory strains in which resistance was artificially induced. Studies showed that resistance involves multiple steps, where overproduction of ornithine decarboxylase and γ-glutamylcysteine synthetase leads to overproduction of trypanothione, which binds to trivalent antimony, and the conjugates are either sequestered within an organelle by ABC transporter MRPA [Brochu *et al.*, 2003; Carter *et al.*, 2003] or extruded from the cell by an efflux pump. In field isolates genes other than described may be overexpressed. In addition, loss of AQP1 allele leads to down-regulation of drug uptake and has been linked to increased resistance to antimony [Mukherjee *et al.*, 2007].

1.8. Conclusion

Leishmaniases affect millions of people, mostly the poorest population groups in developing countries. They are classified among neglected tropical diseases by the World Health Organization. This neglect was due to diversion of efforts to fight major killing diseases: malaria, tuberculosis and AIDS. While

visceral and, to a lesser extent, mucocutaneous leishmaniasis received considerable attention in the last decade, cutaneous forms of the Old World remain largely neglected because they rarely lead to significant disability and rarely, if ever, cause death [Reithinger and Coleman, 2007].

In the mid-1990s it was noted that intralesional treatment of ACL in Aleppo required more injections than in previous years [Douba *et al.*, 1997].

The situation with ACL in Aleppo is unique in several ways: first, it is a long-standing focus with high incidence; second, a state-run control programme has been in place for many years; case detection rate was estimated at 25% in 1991 [Ashford *et al.*, 1993]. And last, the incidence has been increasing during the last decades, reaching peaks about 30,000 cases per year in Syria and exceeding 10,000 cases annually in Aleppo since 2003.

1.9. Objectives of the study

To evaluate the effectiveness of current intralesional antimonial treatment of ACL in Aleppo governorate and determine whether the response of ACL patients is limited by drug sensitivity of the parasite.

1.9.1. Specific aims

1) To quantitatively describe the distribution of time-to-cure among ACL patients throughout Aleppo governorate, who complete a full course of intralesional antimonial treatment.
2) To test whether time-to-cure is significantly associated with parasite sensitivity to antimonials, measured *in vitro* in amastigote-macrophage assay, after adjusting for lesion duration, lesion location, size, number and type pre-treatment, as well as the technique of drug administration.
3) To test whether drug sensitivity decreases during the course of treatment.
4) To evaluate qualitatively the extent to which selection for drug tolerance may be affected by poor treatment compliance (i.e. incomplete courses) and poor administration technique.

5) To identify social and clinical determinants of treatment-seeking and treatment-compliance behaviours.

1.10. Tables

Table 1.1. Species of *Leishmania* that cause disease in man.[*]

Species	Clinical forms	Transmission *(reservoir)*	Other hosts	Distribution
Old World				
Leishmania aethiopica	LCL, DCL	zoonotic *(rock hyrax)*[1]		Ethiopia, Kenya
L. archibaldi (disputed)[2]	VL	anthroponotic (?)	dog	Sudan[3]
L. donovani	VL, LCL, PKDL	anthroponotic		India, Nepal, Bangladesh, Africa, SE Asia
L. killicki (disputed)[4]	LCL	anthroponotic[5]		North Africa
L. major	LCL (rarely chronic and MCL)	zoonotic: *gerbils (Psammomys[6], Meriones), other rodents (Nesokia indica)*[7]		Central Asia, Middle East, N Africa, E Africa
L. tropica	LCL, recidivans chronic	anthroponotic	rock hyrax*, dogs[8], rats	Central Asia, Middle East, SE Asia, N Africa
Old and New World				
L. infantum (chagasi)	VL, LCL	zoonotic: *domestic and stray dogs, wild canids*[6]		Mediterranean basin, Central and South America
New World				
L. amazonensis	LCL, DCL, MCL	zoonotic		South America
L. garnhami	LCL	zoonotic		South America
L. mexicana	LCL, DCL	zoonotic		Central America, Mexico, USA
L. pifanoi	LCL	zoonotic		South America
L. venezuelensis	LCL	zoonotic		N South America
L. (Viannia) braziliensis	LCL, MCL	zoonotic		South America, Central America
L. (V.) colombiensis	LCL	zoonotic		South America
L. (V.) guayanensis	LCL, MCL	zoonotic		South America
L. (V.) lainsoni	LCL	zoonotic		South America
L. (V.) panamensis	LCL, MCL	zoonotic		N South America, S Central America
L. (V.) peruviana	LCL	zoonotic		Peru

DCL: diffuse cutaneous leishmaniasis; LCL: localised cutaneous leishmaniasis; MCL: mucocutaneous leishmaniasis; PKDL: post-kala-azar dermal leishmaniasis; N, S, SE, etc.: north, south, south-east, etc.

[*] Based on a table from a published review [Reithinger *et al.*, 2007], amended to add details about old-world species according to references below.

* Suggested additional reservoir [Jaffe *et al.*, 2004].

[1] [Negera *et al.*, 2008] [2] [Kuhls *et al.*, 2005] [3] [Dereure *et al.*, 2003] [4] [Schönian *et al.*, 2008] [5] [Haouas *et al.*, 2005] [6] [Khiami *et al.*, 1991] [7] [Faulde *et al.*, 2008; Pourmohammadi *et al.*, 2008] [8] [Dereure *et al.*, 1991]

Table 1.2. Number of reported ZVL cases in Syria*

Year	No. cases
1999	55
2000	37
2001	30
2002	38
2003	36
2004	19
2005	18
2007	12†

* [Intersectoral Committee for Vector Control, 2006]

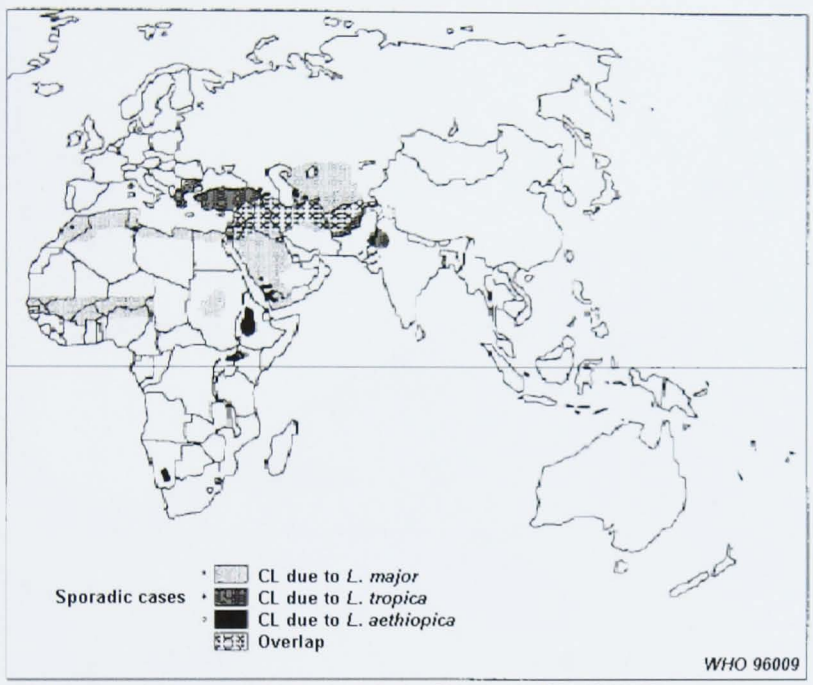
† [al-Shammas, 2008]

Table 1.3. Comparison between ACL and ZCL of the Old World*.

	ACL	ZCL
incubation	long: 2-6 months up to 2 years	short, usually 2-4 weeks
initial appearance	small papule, brownish or flesh-coloured	significant acute inflammatory infiltrate
development	slow	quick
ulceration	3-6 months	1-3 weeks
lymphangitis	rare	frequent
localisation	face > lower extremities	lower extremities > face
epithelialisation	one year or longer	3-6 months
seasonality	primary disease may present round the year	primary disease in summer and autumn
outbreaks	rare	frequent
geography	urban	rural
parasites in lesion	abundant	scarce

* [Koževnikov, 1963; Rodjakin and Sukolin, 1999]

1.11. Figures



Distribution of cutaneous leishmaniasis in the Old World, 1996 (© WHO).

World Health Organization (1997). *Vector Control - Methods for use by individuals and communities*. Prepared by J.A. Rozendaal. Geneva, World Health Organization.

Figure 1.1. Distribution of cutaneous leishmaniasis by causative species in the Old World.

Figure 1.2. Location of Aleppo *(a)* and Syria *(b)*.

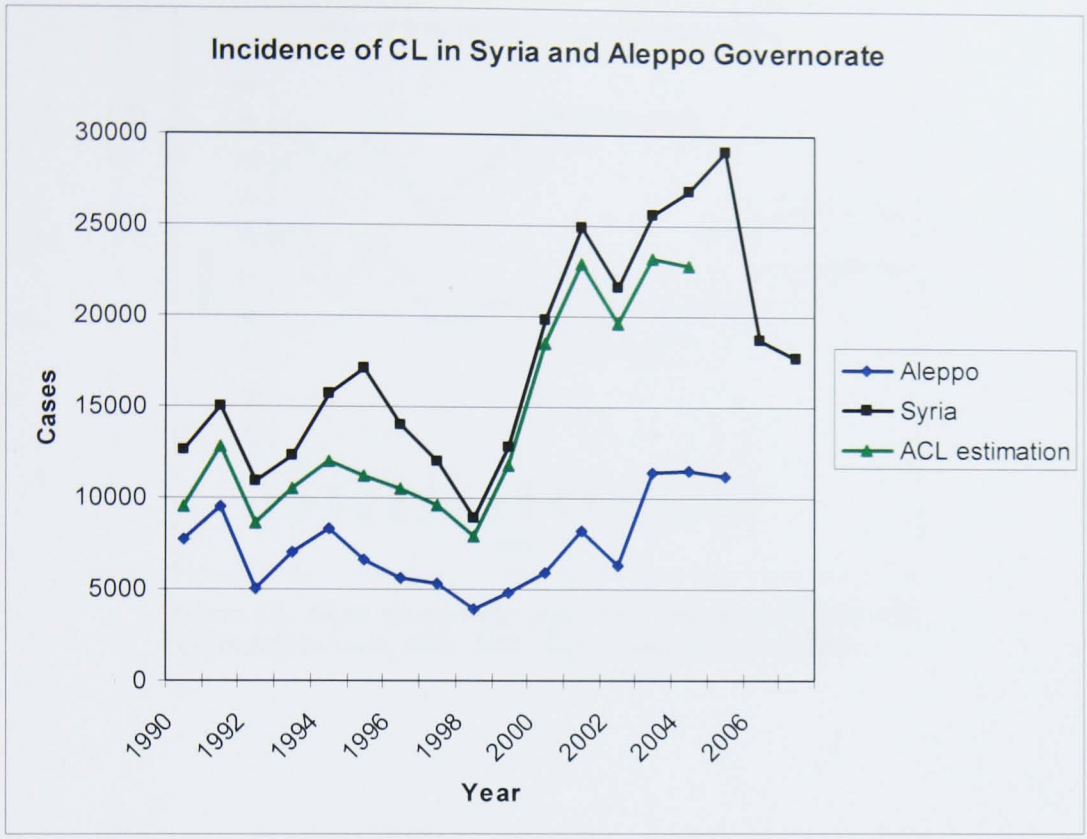


Figure 1.3. Dynamics of numbers of recorded cases of leishmaniasis, 1990–2006, in Syria (black line), Aleppo Governorate (blue line) and estimated numbers of ACL (green line; sum of numbers in governorates where leishmaniasis is believed to be anthroponotic: Aleppo, Hama, Idlib, Lattakia and Tartous Governorates).

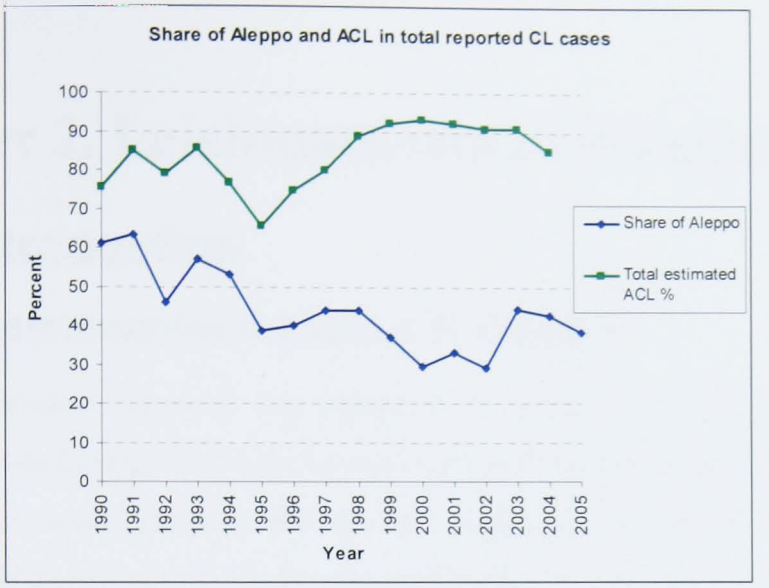


Figure 1.4. Share of reported cases from Aleppo and total ACL (estimated) in Syria, 1990—2005. Total reported cases are 100%.

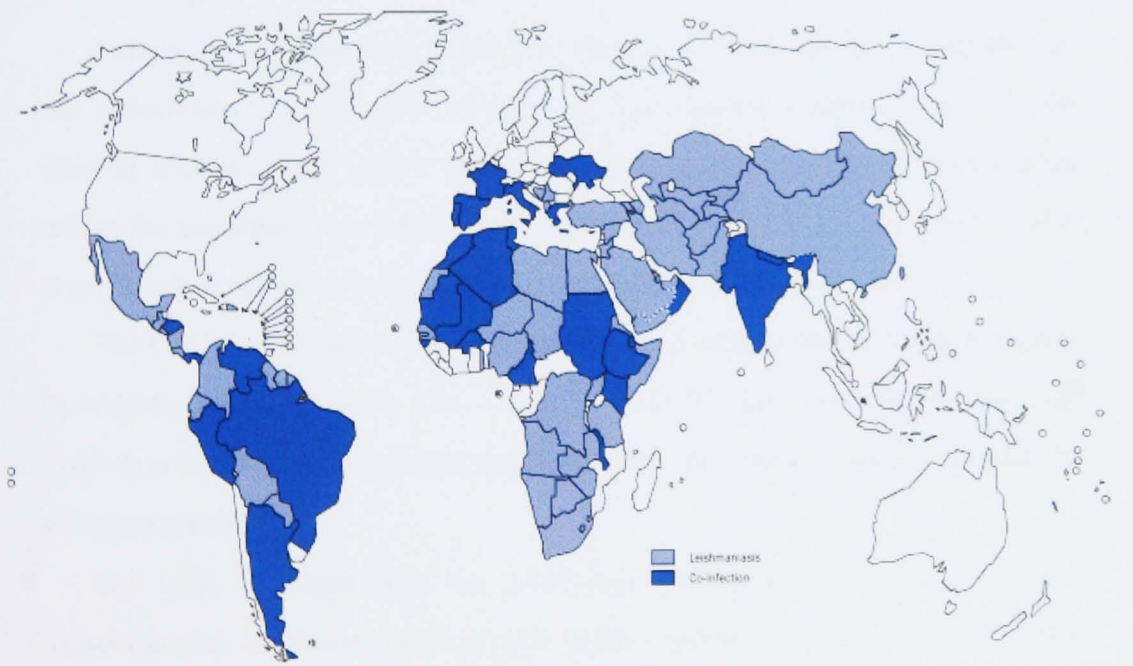


Figure 1.5. World map showing the 34 countries reporting Leishmania/HIV co-infection [World Health Organization, 2008].

Chapter 2. Leishmaniasis in Aleppo

2.1. Introduction

2.1.1. Leishmaniasis control in Aleppo

Leishmaniasis diagnosis and treatment in Aleppo is performed at the Leishmaniasis Control Center (LCC) and at selected primary health care (PHC) centres and health posts in the city of Aleppo and surrounding Aleppo countryside (about 18,500 km^2), coordinated by LCC. In 2004 there were 30 PHC centres diagnosing and treating leishmaniasis, of which 14 (including LCC) were in the city and 16 were in surrounding villages (Figure 2.1), plus seven health posts that only treated cases diagnosed elsewhere: two in the city and five in villages.

Cutaneous leishmaniasis in Syria is diagnosed clinically and confirmed by the detection of amastigotes in a smear from lesion scraping stained with Giemsa and examined under immersion. The patients who have lesions from which the parasites were detected by this method are termed *smear-positive*, and those in whose smears no amastigotes could be seen, *smear-negative*.

The LCC is also responsible for running two campaigns of house-to-house insecticide spraying every year, in May and October, in villages and city districts where the reported disease incidence in the previous year exceeded 1% of the population.

The LCC is responsible for planning when and where to introduce diagnosis and treatment services and puts forward its proposals to Aleppo Directorate of Health. When resources permit, a health care worker from the PHC centre where diagnosis and treatment services are to be introduced is taken for a training course at LCC. Here they are trained in obtaining pathological material from the lesion, preparing smears, and performing smear microscopy, and are taught to correctly administer intralesional treatment. The

training course has no fixed duration but runs until the new worker is deemed competent in performing diagnostic and treatment activities. Necessary equipment and consumables (microscope, microscope slides, Giemsa stain, lancet blades and medicines) are provided by Directorate of Health through LCC.

Health centres differ from health posts in that the former have staff trained to administer intralesional treatment, and the latter are visited once a week by trained staff either from LCC or from a nearby PHC centre that has trained staff. In fact, the term health post can be correct from the point of view of CL control, as in other ways they are usual PHC centres.

Patients with a positive smear made at LCC or a nearby health centre are referred to the health post nearest to their home for their treatment. These patients are reported from the centre that performed the smear.

Some health centres may not have staff fully dedicated for treatment and/or diagnosis of leishmaniasis. In this case they may restrict leishmaniasis clinic time to one or more days a week, usually other than healthy child/vaccination days. In addition, not all treating health centres have the necessary equipment and skill to diagnose CL.

Patients with leishmaniasis confirmed by smear examination start treatment. If a smear examination result is negative there are a number of alternative scenarios:

1. the patient may be referred to LCC where their original smear is read by LCC lab staff, the test may be repeated, and they are examined by a physician who is entitled to diagnose leishmaniasis clinically, regardless of test results, and to refer the patient back to their PHC to receive treatment;
2. in some centres there is a dermatologist who is entitled to confirm leishmaniasis based on clinical presentation of the lesion, regardless of test result, and the patient is treated based on clinical diagnosis (so-called test-treatment, where the patient is re-assessed after receiving a few doses);

3. patients with lesions of short duration (less than a month) may be asked to come back for another smear after two weeks; or
4. in the Specialised Centre for Skin Diseases a patient may have their leishmaniasis diagnosed clinically and administered a test treatment for a few weeks with re-assessment by a dermatologist at each appointment.

Leishmaniasis patients who self-refer to dermatological outpatient clinics in Aleppo University are routinely referred to LCC for diagnosis and further management.

Treatment of leishmaniasis is not compulsory: the patient may choose to interrupt their treatment at any time, without informing the health centre, which they frequently do.

In Aleppo the approved treatment schedule was up to three eight-week courses of weekly intralesional injections with one-month intervals between courses; the patients who, according to the health centre staff judgement, did not heal after receiving these three courses are considered treatment failures and would be referred to LCC for evaluation and further management. In the last years the treatment courses were shortened to six injections, but some centres still administer eight-week courses. When the patient is judged to be cured, they are advised to attend the centre for review after one month, and then at three and six months.

The Specialised Centre for Skin Diseases mentioned above may also apply liquid nitrogen treatment to selected patients, especially those with small lesions on trunk or extremities, and who have not responded to intralesional antimonials.

Depending on how busy health centre staff are, they may either ask a new patient who had his smear taken to wait until the result is available on the same day or come back after an hour or two to start treatment on the same day; or to return on the next workday or the same weekday next week. In the latter case the patient does not start treatment on the day when the smear was prepared. In addition, if a patient presents with a lesion that is complicated by secondary

bacterial infection, then antibiotic ointment is prescribed and the patient is asked to return after a week when the inflammation has subsided. Secondary infection is considered a contra-indication to start treatment and also to reduce the chance of detecting parasites in biological material.

2.1.1.1. Recording

Diagnosis and treatment activities are recorded in health centres as follows:

1. *Laboratory register*: every patient with suspected leishmaniasis who referred to the health centre is recorded. Data fields include: forename, surname, father's name, mother's name and surname (women in Syria retain their father's surname after marriage), age, sex, place of residence, date of referral, number of lesions, lesion duration in months, smear result. The data from this type of register are used in preparing monthly reports. Thus, all the cases are reported only from the PHC centres that diagnose leishmaniasis.
2. *Treatment register*: every patient with confirmed leishmaniasis is entered when they start treatment. The data fields include forename, father's name and surname, age, sex, place of residence, date of diagnosis and dates of treatment sessions. There are three groups of six treatment sessions, separated by two fields for scheduled one-month treatment interruptions. There are also fields marked 'Cure', 'Relapse' and 'Notes'. The date of every treatment session is recorded in the corresponding field of the patient's record. From this register one may calculate how many injections each patient received, and on what dates.
3. *Daily register*: the patients who attend a leishmaniasis clinic for treatment every day are recorded. The data fields include full name, treatment register ID number, number of lesions injected that day and the sequential number of treatment session. This register records the daily number of patients and the quantity of drug used every day.
4. *Patient treatment cards*: every patient undergoing treatment is issued with a card which they keep and bring with them every time they come for treatment. In addition to personal data, the following is recorded: number

and location of lesions, date and result of smear, name of examiner, date of each treatment session, scheduled breaks in treatment course after every six injections, relapse or cure, referral to another centre. Similar cards are used for patients given systemic treatment; since systemic antimonials are given daily for three weeks, no lines for treatment interruption are envisaged. If one card is insufficient, another card can be stapled to it to record additional treatment sessions. At the end of the treatment patient cards are to be returned to the health centre, and at the end of every year they are to be sent to LCC where they are kept for at least five years. If the card is lost, a replacement card is issued, but this only contains personal patient data and the date of diagnosis. Dates of previous injections are not always recorded.

A register is used until the book is full, so every book may span more than one year or even several years if the annual number of patients is low. Registers, when full, are kept at the LCC. One register is in use at any one time, with the exception of treatment registers, as when a new treatment register is started, some of the patients recorded in the previous register are still undergoing treatment.

Demographical, clinical and epidemiological data are extracted and routinely analysed only from laboratory registers.

In practice, data are filled in Laboratory and Daily registers promptly; the same usually applies to Patient cards unless the patient forgot theirs, in which case it would be filled in at the next visit. Injection dates may sometimes not be entered in Treatment registers immediately but postponed until the end of the day or week (sometimes longer), then transferred from daily registers.

Cure is rarely recorded at the time of last injection because there is no clear definition of cure: it is supposed that a patient should not be considered cured until they have finished follow up, which rarely happens.

2.1.1.2. Reporting

Laboratory registers are used to compile monthly recorded incidence reports. At the end of every month all the data are copied manually into separate sheets after sorting by patient's place of residence, and then taken to LCC at the beginning of each month. At LCC the data are entered into a computer database. This custom-made MS-Access database contains macros that can, more or less reliably, identify duplicate records and generate printed summary reports. The practice is relatively new, the database program is updated from time to time, and every year a new database file is created.

Monthly reports are sent to the Directorate of Health which, in turn, sends them to the Directorate of Chronic and Endemic Diseases at the Ministry of Health (MoH). LCC also faxes monthly reports directly to the Directorate of Chronic and Endemic Diseases. Directorates of Health reports include only smear-positive patients; the number of smear-negatives is mentioned separately (in Aleppo). Smear-negative patients are not included in annual reports compiled by MoH.

2.2. Objectives

5. Describe the demographic and clinical characteristics of cutaneous leishmaniasis in Aleppo.
6. Describe the seasonal variation in the number of cases at PHC centres.
7. Determine whether the response to treatment is influenced by either demographic or clinical (lesion number and duration at presentation) characteristics of a case.
8. Determine if response to the treatment is influenced by the health centre where treatment is carried out or the month of presentation.

2.3. Materials and methods

Four data sources were used in the analysis in this chapter:

9. The Main database, based on laboratory registers obtained from LCC which contained data on more than 14,000 cases of cutaneous leishmaniasis recorded from 19 centres during 2005.
10. To obtain treatment data, patient treatment cards of cases whose treatment started in 2005 were obtained from the LCC. These were matched with the Main database, and the treatment data were entered in a table linked to the Main database table. Data recorded were: number of treatment sessions, dates of first and last recorded injection, date of cure (if recorded in the card), scheduled treatment breaks, relapse, referral to another centre. Only 528 cards from 15 centres were available.
11. Treatment registers covering the first three months of 2005 were requested by the LCC from all centres, but were available from only 13 centres. The data concerning about 2000 patients who started treatment in the period from January to March 2005 inclusive were entered into an MS Excel workbook. The data recorded were patient's name, age, sex, place of residence, reporting health centre, number of lesions at presentation, dates of injections, total number of injections, date of last injection, and available data about cure or relapse.
12. Statistical Abstract 2006 by Syrian Central Bureau of Statistics [Central Bureau of Statistics, 2007] was used. The following data were reported: population distribution by age group and sex for Syria and population by sex for each governorate. Data from census of 2004 were used for comparisons.

All the databases may have intrinsic biases. Comparing them may give insight into some of these biases.

13. All four data sources incorporate *demographic data*, permitting performing comparisons of distribution by age group and sex;
14. The three 'leishmaniasis' data sources (numbered 1, 2 and 3 above) incorporate *baseline* data, i.e. lesion duration at presentation and number of lesions at presentation, either counts or mean, median, etc. values by age, sex, health centre (and lesion duration at presentation by number of lesions). Data on lesion duration are not available in treatment registers.

15. In addition, month of presentation and smear result can be accounted for (data sources 1 and 2 because treatment registers only span January to March). Since therapy is usually initiated based on smear result, higher positivity rate can be expected in Patient cards sample.
16. between two *clinical* data sources (2 and 3): clinical data, i.e. relationship between baseline variables (age, sex, health centre, number of lesions) and treatment duration (mean or median time or number of injections) as outcome variables.

The data in MS Excel spreadsheets were analysed using SPSS version 16). Data from MS Access databases were exported to MS Excel first, then analysed as above. Graphs were prepared in MS-Excel.

2.4. Results

2.4.1. Demography

The first set of tests was designed to determine whether age and sex distribution between general population (that of Syria and Aleppo) is different from recorded leishmaniasis population and datasets from treatment registers and patient cards.

The Main dataset contained demographic and clinical presentation data on 14,368 patients who referred to PHC centres in Aleppo in 2005 and were examined by obtaining a smear. The Treatment dataset contained data on the number of treatment sessions for the 2145 patients who started their treatment from January to March 2005. The patient cards dataset contained demographic and clinical presentation data and the treatment sessions for 498 patients who started their treatment during 2005 and returned the patient cards upon completion of treatment.

2.4.1.1. Demography — Results

2.4.1.1.1. Age distribution

Raw frequencies

Counts of patients at every age were performed. The presence of attractors, i.e. values that are consistently higher than expected, was noted, especially in the two larger datasets (see Figure 2.2).

Attractors in our data occur consistently every five years, at multiples of five. They probably occur because patients and/or health workers tend to round the age (to nearest five years). In the Main dataset they are clear from the age of five, while in Treatment dataset they can be suspected from the age of 20 and become obvious from 25 years. They were not prominent in Patient Cards dataset, probably due to low number of patients of some ages.

There were statistically significant differences between the median age values among the three datasets (shown in Table 2.1): $p<0.0001$ (Kruskal-Wallis H test).

Age group comparisons

Patient age was categorised as in the census that uses five-year age groups (Figure 2.3) to allow comparisons. Count distribution tables were produced in SPSS. In the Main dataset and Patient cards no patients were recorded as under one year of age; probably, ages <1 year were recorded as 1 year. For this reason, age group of <1 year was merged with the next group to form 0–4-year-old group. This also has the advantage of better comparability because the age groups in the census span five years (except the last, 65+, group).

The age groups followed the classical demographic pyramid in the census, whereas in leishmaniasis patients the age group 0-4 years was smaller than the next, 5-9 years, age group, and the population in general was younger (Figure 2.3).

Syrian population according to 2004 census:

Mean and percentile values of age distribution are not stated explicitly in the census tables but percentiles can be inferred: the lower quartile is at 9 years, the median is about 19 years, the upper quartile is at 33 or 34 years. This is summarised in Table 2.1.

Age quartiles in leishmaniasis datasets are lower compared to census data. In the Treatment dataset, the mean and age quartiles are 1-2 years lower compared with the Main dataset (recorded leishmaniasis population) data. This shows younger patients are more likely to start treatment. The data in the Patient cards dataset seem closer to treatment register statistics than to the Main dataset.

2.4.1.1.2. Sex distribution

While in Syria and Aleppo governorate there is a slight male predominance in the population, among leishmaniasis patients from all datasets females predominate (Table 2.2).

The sex distribution of leishmaniasis cases was similar in the different datasets ($p= 0.71$, Pearson's χ^2 test). However, it was significantly different from the general population in Aleppo: $p<0.0001$ for the Main dataset, $p=0.015$ for Treatment registers dataset and $p=0.0002$ for Patient cards dataset (χ^2 test).

To assess the sex distribution by age group, sex was cross-tabulated with age groups in the main dataset; in the three younger groups, but not in the older ones, the sex ratio was fairly equal and close to that of general population. Collapsing age into two age groups: "14 years and younger", and "15 years and older", M:F ratio in the younger group was 50.4:49.6. The same ratio was also calculated for groups 0–14 years from census data for Syria. It was 50.3:49.7, i.e. the same ($p=0.91$, χ^2 test), while the difference between the two age groups was highly significant ($p<0.0001$, Pearson's χ^2 test). This breakpoint was selected for two reasons: first, it represents a breakpoint in census age groups, and thus

allows one to calculate the relevant values from census data for comparison; second, it is close to the median age in the Main dataset.

This means the inversion of the sex ratio occurs in the older age group only, while in patients of 14 years and younger it does not differ from the general population.

Proportional risk ratio was explored in census age groups. To this end, percent of the patients belonging to either sex in each age group was calculated (or taken from census data). Census data percentages were used as the denominator. Percent of males or females in a given age group in a given dataset was divided by the percentage of the corresponding age and sex group in the census (Figure 2.4).

2.4.2. Presentation data of leishmaniasis patients

2.4.2.1. Aims

1. To describe presentation variables of leishmaniasis patients in Aleppo and explore differences between the three available datasets.
2. To describe factors which may affect each of the presentation variables.

2.4.2.2. Methods

In SPSS, frequencies, mean, median and relevant quantile values of the variables were generated. Frequencies were first calculated raw then grouped in different ways until the most appropriate grouping was found. Cross-tabulation or logistic regression was used for categorical variables.

Presentation variables examined were number of lesions, lesion duration and smear result. Explanatory variables were age and sex (for all dependent variables), number of lesions (for lesion duration and smear result), lesion location (for lesion duration and month of appearance; patients with one lesion or those with lesions in one location were analysed) and lesion duration for smear result.

Lesion duration and smear result data were not available for Treatment dataset.

2.4.2.3. Presentation data — Results

2.4.2.3.1. The reporting health centres

The data were available from nineteen health centres in the Main dataset, thirteen in Treatment dataset and fifteen in Patient Cards dataset. These centres and the number of cases available from each centre in each dataset are listed in Appendix 1.

Table 2.3 displays the distribution of patients by place of residence between urban and rural health centres. More than one-quarter of patients in urban health centres lived in rural Aleppo. Rural health centres served almost exclusively rural residents (99.8%). Overall, about forty percent of all patients recorded in 2005 came from rural areas.

2.4.2.3.2. Distribution of cases by month

This can be assessed reliably from the Main dataset only, as Treatment dataset included only cases starting treatment between January and March 2005, and patient cards for last months of 2005 were not available for analysis because many of them were not returned to LCC.

Figure 2.5 shows percentages of patients from the Main dataset (squares) and Patient Cards (circles) presenting every month.

Mean monthly percent (dotted line) is $100 \div 12 = 8.3$. Months above the mean value are December to May inclusive.

2.4.2.3.3. Number of lesions at presentation

Frequencies

About half of patients presented with one lesion, 53.3% in the Main dataset, 48.9% in the Treatment dataset and 56% in Patient cards dataset, and the proportions of patients with two lesions were similar (Figure 2.6). Treatment Registers dataset has the lowest proportion of patients with one lesion and,

reciprocally, higher proportions of >2 lesions; Patient Cards dataset is *vice versa* (Figure 2.6). The differences between the datasets are statistically significant: p=0.001, Pearson's χ^2 test.

Distribution by age

The mean and median numbers of lesions in younger and older patients were significantly different, with Treatment dataset showing most and Patient Cards dataset showing least difference (Table 2.4).

Distribution by sex

In all datasets mean values for the number of lesions was marginally but consistently higher among females. As for the medians, they were equal to one in all groups except females in Treatment dataset, where it was 2. Nevertheless, the differences were statistically significant only in the Main and Cards datasets, where the median values are seemingly equal (Table 2.5).

Distribution by month of presentation

Only the Main dataset was analysed because Patient Cards contain fewer subjects towards the end of the year and the Treatment dataset spans only the first three months of 2005. Patients presented with more lesions than all-year average during the first three and the last three months of 2005. The month of appearance was calculated by subtracting the reported lesion duration from month of presentation. Numbers higher-than-average for the whole year appear from August to November (Figure 2.7). When the patients were categorised into two groups according to month of appearance (August to November vs. other months), the mean number of lesions was 2.46 and 2.00, and the medians, two months and one month, respectively. These differences were statistically significant, p<0.0001 (Mann-Whitney U test).

Logistic regression

Number of lesions, grouped into two categories, "1 or 2 lesions" and "3 lesions or more", was examined by binary logistic regression in the Main

dataset using a univariate model with one explanatory variable at a time, then a multivariate model that included all the variables for which significant relationship with outcome was demonstrated. The results are presented in Table 2.6.

In both univariate and multivariate analysis all the variables tested had a statistically significant impact on the odds ratio. The change in odds ratio between univariate and multivariate models was marginal suggesting that the factors are independent.

2.4.2.3.4. Lesion location

Frequencies

This analysis was first restricted to patients with one lesion because in patients with multiple lesions it would not be possible to distinguish the effects of lesion location. These patients represent 53.3% of all recorded leishmaniasis patients. The results are shown in Figure 2.8. Most patients presented with lesions of the face, followed by upper, then lower extremities.

Distribution by age and sex

After preliminary analysis, patients with one or more lesions in one location were analysed (n=12,176; 84.7%) and, additionally, all patients, including those with multiple lesion locations, were categorised into those who have any lesions on face and those who had no lesions on face. The data for the three most common lesion locations by sex and age group are shown in Table 2.7 and Table 2.8, respectively.

There were significant differences in lesion locations between age groups and sexes in patients with one lesion. To calculate statistical significance, the locations in patients with one lesion (n=7650) were categorised as *Face* and *Non-Face*, *Hand* and *Non-Hand*, *Leg* and *Non-Leg*, and cross-tabulated. All the differences were statistically significant, except sex differences for lesion location on the lower extremities.

Similar results were obtained when patients with one or multiple lesions in one location (n=12,176) were compared, and when the patients with multiple lesion locations with any lesions on face were compared to those with no lesions on face.

Distribution by reported month of lesion appearance

In the Main dataset, the date of lesion appearance is calculated by subtracting the patient-reported lesion duration from the date of patient referral to the centre. Month of reported lesion appearance was extracted, and then the cases with lesion durations of 12 months and longer were excluded because after long periods the patients tend to round up the duration of their lesions, so the data about the month of appearance become unreliable.

The distribution of the three most common lesion locations (face, upper and lower extremities that constitute more than 97% of total) by month of appearance was examined in this group. Figure 2.9 shows the percentage distribution of the three most common lesion locations for each month (points for each month add up to 100%).

Month of appearance was re-categorised into three categories of four months each (January to April, May to August and September to December) and cross-tabulated with the three locations. The differences are significant, $p<0.0001$ (Pearson's χ^2 test). The proportion of face lesions diminishes in the warm months due to the rise of the proportions of other locations.

Logistic regression

Lesion location, categorised to face/non-face was examined by binary logistic regression in the Main dataset using a univariate model with one explanatory variable at a time, then a multivariate model with all the explanatory variables included. The results are presented in Table 2.9. The changes in odds ratio between univariate and multivariate models were marginal suggesting that the factors are rather independent.

All the tested variables significantly affected lesion location. Females were slightly less likely to have lesions on the face, older patients much less likely than younger ones, and, compared to the period between January and April, lesions on the face were less likely in the hottest months (May to August) and slightly more likely in September to December.

2.4.2.3.5. Lesion duration at presentation

Frequencies

About two-thirds of patients reported lesion duration of 2 months or less, and about 90% of 5 months or less. Mean lesion duration was 2.88 and 2.71 in the Main and Cards datasets, respectively, and the median lesion duration was 2 in both datasets (p=0.096, Mann-Whitney U test) and in all the examined subgroups.

The results by subgroup are presented in Table 2.10 and described in the text below.

Distribution by age

The lesion duration in the older age group (15+) is higher than in the younger. Difference in mean values in the Main dataset is estimated 24 days *vs.* less than two weeks in Patient Cards dataset, and both are statistically significant: p<0.0001 for the Main dataset, p=0.025 for Patient Cards dataset (Mann-Whitney U test).

Distribution by sex

Males seem to report slightly longer durations of lesions than females. The difference is no more than one week, not statistically significant: p=0.126 for the Main dataset, p=0.305 for Patient Cards dataset (Mann-Whitney U test).

Distribution by number of lesions

Number of lesions was categorised into 2 groups: "1 or 2 lesions" and "three or more lesions".

The patients with more lesions tended to present slightly later in time, and the difference was more pronounced in the Cards dataset (more than half a month *vs.* less than a week in the Main dataset). Both are statistically significant: $p<0.0001$ for the Main dataset, $p=0.001$ for Patient Cards dataset (Mann-Whitney U test).

Distribution by lesion location

The relationship between the three most common lesion locations (face, hand and leg) and lesion duration was examined in patients who had one lesion, in patients who had their lesions (one or more) in one location, and all the cases after categorising them into those who had at least one lesion on the face, regardless of whether they had any other lesions, and those who had no lesions on the face.

In Table 2.11 the mean reported lesion durations for the three most common lesion locations are compared. Lesion location on the face was associated with a shorter reported lesion duration at presentation compared to other lesion locations. The difference is estimated 17–19 days, and is statistically significant.

Figure 2.12 is a graphical illustration of the distribution of reported lesion duration at presentation, relative to all patients in location group (points for each location add up to 100%). The patients with facial lesions tend to present earlier than those with no lesions on the face.

Distribution by month of presentation

Mean lesion duration is higher than the average for the year from March to September inclusive (Figure 2.13). Median values are equal to 2 throughout all months, yet the differences are significant ($p<0.0001$ by Kruskal-Wallis test). This represents a three-month shift past month of presentation curve (see page 64), assuming roughly equal disease cycles (i.e. assuming that current December can be substituted for last-year December). The data presented are for the Main dataset. In the Patient Cards dataset the relationship appeared random due to small number of patients, and is not shown. When the patients

in the Cards dataset were categorised by month of presentation into two groups according to the data from the Main dataset, i.e. to those who presented from March to September and those who presented in the other months, the mean lesion duration at presentation was 2.91 months in the former and 2.26 months in the latter group. This difference was not statistically significant, p=0.156 (Mann-Whitney test).

The trend in the Main dataset is the gradual increase in lesion duration from the start of usual reported appearance of lesions in the end of summer, consistent with increasing length of time from transmission season: the patients who present in autumn and winter have acquired their lesions during the transmission season, before October, so the lesion duration increases. Starting from June, the reported lesion duration decreases. This is probably due to patients with short lesion durations whose lesions appeared in the next transmission season.

Differences between health centres

Mean and median lesion duration was calculated by reporting health centre. The mean values ranged between 2.2 months for Atareb and 3.9 months for Salah al-Din (Figure 2.14). The median values were equal to 2 months in most centres, with the exception of Efrin, Halab al-Jadidah and Salah al-Din, where they were 3 months.

The centres were categorised into those with short (n=7629) or long (n=6739) mean reported lesion duration, with the breakpoint being the mean lesion duration for the complete dataset (2.9 months). The mean lesion duration was 2.4 months in the former group and 3.4 months in the latter. The median was 2 months in both groups but the differences were statistically significant, p<0.0001 (Mann-Whitney test).

It was noted that of the four rural centres, three belong to the former category, so the centres were also categorised into urban (n=11,787) and rural (n=2,581). The mean lesion duration was 3.0 months in urban centres and

2.3 months in rural centres. The median lesion duration was 2 months in both but the differences were statistically significant, $p<0.0001$ (Mann-Whitney U test).

2.4.2.3.6. Smear result

Frequencies

These are only available for Main and Patient Cards datasets, and are summarised in Table 2.12.

Proportion of smear-positive patients is significantly higher in Patient cards dataset compared to the Main dataset: $p<0.0001$ (Pearson's χ^2). This is to be expected because the positive smear result is the main diagnostic criterion for CL in Syria. The Main dataset comprises all the patients who were referred or self-referred to health centres and had their smear taken, regardless of whether or not they were treated later. Patient cards dataset represents the patients who started treatment, thus smear-positive patients are selected into this group.

Distribution by age

A clear decline in percentage of positive smear results with increasing age was noted in the Main dataset but not in the Cards dataset (Figure 2.15). The differences between age groups were statistically significant for the Main dataset ($p<0.0001$, Pearson's χ^2) and not significant for the Cards dataset ($p=0.297$, Pearson's χ^2).

The trend was analysed by binary logistic regression with age as continuous variable which gave a low but highly significant ($p<0.0001$) B value of -0.014 ± 0.001, i.e. a 1.4% fall in positivity rate for every additional year of age.

The data were also analysed according to ten-year age groups. The results (Table 2.13) show declining odds ratio with increasing age, and all the differences are highly significant. In those aged over forty the odds ratio is roughly one-half compared to the youngest age group.

The absence of this trend in Patient Card dataset (binary regression: B=0.001±0.011, p=0.925) can be explained by the fact that the patients are selected for treatment based on positive smear result.

Differences by sex

No significant differences between sexes were detected in either dataset.

Differences by number of lesions

This was uniform across number of lesions categories in the Main dataset and with random fluctuations in the Cards dataset, especially in the groups with more lesions that contained few patients each. When divided into two groups, of "1 or 2 lesions" and "3+ lesions", the results were the same for both.

Differences by lesion location

The patients who had any lesions on the face were more likely to have a positive smear result compared to those who had no lesions on the face, 80 vs. 76.4% ($p<0.0001$, Pearson's χ^2).

Differences by lesion duration

In the Main dataset the smear positivity was higher than the total average (i.e. that for the whole dataset) in lesions of 2, 3 and 4 months' durations. In the Cards dataset the numbers of patients of lesion duration of 5 months fell below 20, so lesion duration was re-categorised into less-than-one, 1, 2, 3, 4 and 5+ months. Above-average positivity rates were in lesion durations of up to 3 months (Figure 2.16).

The mean and median lesion duration in smear-positive and smear-negative patients was explored. The median lesion duration was 2 months in both categories of both datasets. The difference between the mean durations was about 19 days in the Main dataset (2.74 *vs.* 3.37 months) and about 38 days (2.59 *vs.* 3.85 months) in the Cards dataset. The differences between the medians were statistically significant ($p<0.001$ for the Main dataset and 0.004 for the Cards dataset, Mann-Whitney U test).

The relationship was also analysed by binary regression with less-than-one-month duration as reference category and six lesion duration categories. The odds ratio peaked to more than 2 at 2 month lesion duration then declined to about one in the longer-than-five-month category but these differences were not statistically significant.

When lesion durations were divided into three roughly equal groups: "one month or less" (n=4175), "two months" (n=5150) and "three months or more" (n=5043), the patients who reported lesion duration of 2 months were significantly more likely to have a positive smear result (odds ratio = 1.39, 95% CI = 1.26–1.54, $p<0.0001$) compared to patients with lesion durations of one month or less. The patients with the longest reported lesion duration (three months or more) were less likely to have positive smear result than patients with lesion durations of one month or less: OR = 0.92, 95% CI = 0.84–1.01, $p=0.085$, the difference not statistically significant), and patients with lesion durations of two months were significantly more likely to have a positive smear result compared to those with lesion durations of three months or more (OR = 1.51, 95% CI = 1.37–1.66, $p<0.0001$).

Differences by health centre

This was calculated for the Main dataset only (Figure 2.17). Marked differences for smear positivity between the centres can be noted, with minimum of 58.1% in Salah al-Din. Numbers of reported cases were high, two lowest count of patients were 55 in Haydariyyeh and 96 in Efrin.

Smear positivity rate was 82.4% in rural health centres *vs.* 77.0% in urban health centres, and this difference is statistically significant, $p<0.0001$ (Pearson's χ^2 test). However, this may be confounded by lower mean reported lesion duration in rural health centres (*see* Differences between health centres *in* Lesion duration at presentation, p. 70), so a multivariate analysis including the variables that were significant in univariate analysis above (age, lesion duration and health centre location) was performed. The results by age group were

essentially the same, the maximum difference in odds ratio between univariate and multivariate analysis did not exceed 0.01 (Table 2.13). For lesion duration, the patients who reported lesion duration of 2 months were significantly more likely to have a positive smear result (odds ratio = 1.43, 95% CI = 1.29–1.58, $p<0.0001$) compared to patients with lesion durations of one month or less. The patients with the longest duration (three months or more) were as likely, compared to patients with lesion durations of one month or less, to have positive smear result: OR = 1.01, 95% CI = 0.92–1.11, p=0.837).

Some rural residents might either not have access to CL diagnosis in their local PHC centre or be unaware that such diagnosis is available, so they would refer to urban health care centres or the LCC. Smear result positivity was analysed according to patient's reported place of residence (urban vs. rural), and rural residents (n=5,777, or 40.2% of total) had a marginally higher smear result positivity rate (78.4%) than urban residents (77.7%), and this difference was not statistically significant (p=0.329, Pearson's χ^2 test).

2.4.3. Treatment response data

2.4.3.1. Aims

The aims of this part of the study were:

17. To describe the distribution of clinical outcome data and explore the differences between the two available datasets.
18. to explore the relationship between outcome and the demographic and presentation explanatory variables.

Four outcome variables were originally expected: time to cure, relapses, number of treatment sessions (injections, or doses) and treatment duration calculated as [date of last dose] – [date of first dose]. The latter gives the result in days; it was recalculated to weeks by taking integer of [days ÷ 7]. Another recalculation was done, the integer of [(days+3) ÷ 7] to take mid-week points, and the latter variable is used in all calculations.

Relapses could not be reliably estimated in most cases (see the next section), leaving two outcome variables: number of doses and treatment duration. Outcome variables will be discussed in parallel: for each explanatory variable, number of recorded doses, treatment duration and time to cure will be reported, the latter for Patient cards only.

The explanatory variables were categorised when convenient as described previously. In addition to the explanatory variables mentioned above, differences by treating health centre were explored.

2.4.3.2. Treatment response data — Results

2.4.3.2.1. Frequencies

Cure and Relapse fields

Cure date is rarely recorded, although it is more likely to be present in the Cards sample, probably because it over-represents the patients who were considered cured and whose cards were requested back from them. In the treatment registers the Cure field is usually left empty. In some cases the word *Cure* is recorded without the date, which will be referred to as *"Fact of cure"*, and in rare instances, the date of cure. The date of cure was recorded for seven patients (0.3%) in the Treatment dataset, and for 71 patients the word *Cure* was filled (without the date). In the Cards dataset the date of cure was recorded for 189 patients (38%).

Thus, in most instances it is impossible to tell whether or not a patient was cured. We can only depend on recorded treatment sessions to calculate the number of doses and treatment duration. A selection of 38% of patient cards, which are initially biased towards the beginning of the year and by patient self-selection, are not a reliable source to estimate time to cure; the seven-patient (0.3%) selection from treatment registers is probably even less reliable. No analysis of this variable in Treatment registers will be attempted, but the 189 patients from Patient cards dataset merit investigation despite the likely biases.

Relapses were not encountered in patient cards, probably because either such patients would still undergo treatment, and total treatment duration would be too long to finish within 2005, or because the patients who returned their cards would be issued a new one when they refer for treatment of a relapse. In Treatment registers 45 counts of relapse exist, two of them include a precise date. This variable will not be analysed.

Variation between primary health care centres

Proportion of cards of the patients who started their treatment during 2005, returned from different health centres by end of April 2006, was estimated by dividing the number of available patient cards by the number of reported smear-positive patients from the same centres in 2005. The denominator was selected as a proxy for patients who started treatment and, hence, had cards issued to them. In reality, some smear-positive patients may refuse to start treatment, and some smear-negative ones may start treatment based on clinical examination only. The results are presented in Figure 2.18.

Salah al-Din centre has the highest card-return rate. It is the centre with the lowest smear positivity rate, which may mean that considerable proportion of patients start treatment despite negative smear results, whose cards are also counted, thus increasing the calculated proportion. Twenty-five percent of cards from this centre belonged to smear-negative patients, the highest proportion among centres from which more than 20 cards was available. Nevertheless, this centre still had the highest proportion of cards returned to LCC when total number of patients was used as denominator (data not shown).

Proportion of cards where cure was marked was calculated. Total number of available cards was used as denominator.

Health centres are sorted by the number of available cards, indicated by colours and patterns in Figure 2.19 (green, dotted: ≥60; blue, diagonal: 30–59; yellow, horizontal: 20–29; and pink, checkerboard: <20). Salah al-Din centre, the

one that had the highest proportion of cards returned, has the lowest proportion of filling in the date of cure.

2.4.3.2.2. Description of treatment data

Number of recorded doses

The data are summarised in Table 2.14. Two hundred and seventeen patients (10.1%) in the Treatment registers dataset received only one dose, and the mode was six doses (228 patients, 10.7%). This reflects the six-dose treatment courses adopted in most PHC centres. There is a suggestion of a second peak at 8 doses, possibly because some PHC centres used 8-week courses. The maximum number of doses recorded in the Treatment registers was 24 (Figure 2.20).

The Patient cards dataset contains about 500 patients. Only eight values exceed 20, and only six of them were ≥30. This explains the more random appearance of the graph. A striking difference from treatment registers is lower number of patients receiving few doses and a clear mode at twelve doses, with smaller peaks at eight, then six doses.

The maximum number of recorded injections was 40. This may be because when a card is full, another card can be attached to it where additional doses are recorded, while in the Treatment registers there is no room to record too many doses because every record is represented by one line.

The median number of recorded doses was six in the Treatment dataset and ten in Patient cards. This difference is significant: Mann-Whitney U test, $p<0.0001$.

Duration of treatment

Treatment durations in Treatment registers distribute along a wider range than the number of recorded doses, while in Patient cards the range was only slightly wider than number of doses.

In Treatment registers dataset 224 patients (10.5%) had duration of 0 weeks (i.e. one injection only). A clear peak occurred at 5 weeks which corresponds to

the sixth dose. Low corresponding values in Patient Cards dataset may indicate early patient drop-out (Figure 2.21). The median duration of treatment was 7 weeks in Treatment dataset and 12 weeks in Patient cards, and the difference is statistically significant ($p<0.0001$, Mann-Whitney U test).

In the Patient Cards dataset there was a significant difference in the number of recorded doses and treatment duration between the patients with date of cure recorded on the card and those whose date of cure was not recorded. The median number of recorded doses was 10 and 11 ($p=0.019$, Mann-Whitney U test), and the median treatment duration, 11 and 13 weeks, respectively ($p=0.01$, Mann-Whitney U test).

Time to cure

Time to cure was calculated in weeks in the same way as for treatment duration, i.e. date of first injection was subtracted from date of cure, giving number of days, then 3 was added to the result to take mid-week points, and the integer of the result divided by seven was taken.

Figure 2.22 shows the raw distribution of weeks until cure, in percent, in the subset of patient cards where the date of cure was recorded (n=189).

This distribution is shifted to the right (the percentile values are higher) compared to treatment duration, probably reflecting the cases in which cure was diagnosed after a scheduled or non-scheduled break. Only 4 of 189 patients have time to cure of six weeks or less, and the median time to cure is 18 weeks.

2.4.3.2.3. Differences by age

The difference in the median number of recorded doses and median treatment duration between younger and older patients in both datasets is equal to one dose or one week, respectively. This difference is statistically significant in the Treatment dataset only. In the Patient Cards dataset the differences in the number of doses, treatment duration and time to cure between older and younger age groups are not statistically significant (Table 2.15).

2.4.3.2.4. Differences by sex

In the Treatment dataset the median numbers of recorded doses and treatment duration is less for males, with difference of one dose or week, respectively, is significant. In the cards dataset the median number of recorded doses is ten for both sexes but females seem to have longer duration of treatment and time to cure (the latter difference being two weeks). However, the differences in the tested parameters in Patient Cards dataset were not statistically significant (Table 2.16).

2.4.3.2.5. Differences by number of lesions at presentation

Number of lesions at presentation was grouped into two categories: "1 or 2 lesions" and "3 lesions or more". Although the median values were consistently higher in the group with three lesions or more in both datasets, the differences were not statistically significant, except for the number of recorded doses in Patient Cards dataset (Table 2.17).

2.4.3.2.6. Differences by health centre

Treatment registers were available from 13 health centres, cards from 15 centres (four of them <20 cards each) and both registers and cards from 7 health centres, and of these, two centres had very low number of cards (<10). For treatment registers the lowest count of patients from a health centre was eighty-five.

The patients from the seven health centres represent 67.6% of the cases in the Treatment dataset and 47.8% of the cases in the Patient Cards dataset.

Number of doses

The differences in median number of doses between the two datasets (Figure 2.23) are statistically significant for three of the five PHC centres with patient numbers >20 in the Patient Cards dataset, p=0.001 for Halab al-Jadidah and <0.0001 for Bab and LCC, and not significant for Nayrab (p=0.744) and Salah al-Din (p=0.068), Mann-Whitney U test. The differences between centres within each dataset were statistically significant (p<0.0001, Kruskal-Wallis test).

Duration of treatment

The differences between the median treatment duration between the two datasets (Figure 2.24) were significant for LCC and Salah al-Din centres (p<0.0001, Mann-Whitney test). The differences between centres within each dataset were statistically significant (p<0.0001, Kruskal-Wallis test).

Time to cure

The distribution is presented in Figure 2.25. The differences between the centres are statistically significant, p=0.002 (Kruskal-Wallis H test).

2.4.3.2.7. Difference by lesion duration at presentation

Data on lesion duration were not available in the Treatment dataset, so the analysis is restricted to Patient Cards. Lesion duration was categorised into three groups: one month or less, two months, and three months or longer. The categories contained fairly equal numbers of patients. The results are shown in Figure 2.26.

Patients who reported lesion durations of one month or less required more doses and had longer treatment duration and time to cure compared to those who reported lesion durations of two months or longer. The differences were statistically significant for number of doses and treatment duration (p=0.0002, Kruskal-Wallis H test) but not for time to cure (p=0.137).

The statistical significance for any of the outcome variables did not change when lesion duration was compared between one month or less, on one hand, and each of the other two durations alone, on the other.

The differences between lesion durations of two months *vs.* three months or longer were not statistically significant for any of the three outcome variables (p=0.369, 0.134 and 0.823, respectively, Mann-Whitney U test).

2.5. Discussion

Analysis of the data showed marked differences between the data sources used, and differences between the general population and the population of leishmaniasis patients in Aleppo as represented by the Main dataset.

2.5.1. Observed differences

2.5.1.1. Demographic variables

It is postulated that differences between datasets are partly due to the nature of each dataset: laboratory registers contain all patients with suspected leishmaniasis, which means not all of them will have leishmaniasis confirmed and start treatment; an entry in the treatment register appears when the patient receives their first injection, which implies they had their diagnosis confirmed based on smear result or on clinical examination alone and decided to start treatment; and, as only cards that were returned by patients are available for analysis, this dataset represents patients who completed their treatment (or, at least, is biased in this direction compared to treatment registers). This does not exclude other possible biases due to non-randomness of both clinical datasets, at least because they represent a non-random selection of health centres.

A typical patient with cutaneous leishmaniasis in Aleppo is a child or adolescent with one non-ulcerated lesion who presents within two months after the lesion has appeared.

The median age of leishmaniasis patients is six to eight years lower than that of the general population. In addition, there were statistically significant

differences between the three datasets (Figure 2.3 ; Table 2.1). This difference may be real, consistent with long-standing endemicity of a disease that confers some level of immunity; in this case residents are more likely to acquire symptomatic or asymptomatic infection and, hence, acquire immunity at younger age (probably, with natural subclinical booster inoculations later). But the immune response of patients in the younger age group may also contribute to these differences, and may be related to prepubertal hormonal background, as evidenced by proportional risk ratios of CL patients: while the group below 14 years of age is characterised by male-to-female ratio similar to that in the general population, in the 15+ age group this ratio is inverted (see Sex distribution, p. 62). On the other hand, a behavioural explanation should be considered: it is possible that older patients make the decision to seek diagnosis and start treatment themselves, while in case of younger patients, parents take the decision; and in older age groups females may be more likely to seek treatment for potentially cosmetically disfiguring condition than males, and hence, are more likely to put up with painful injections.

Age distribution of all leishmaniasis patients from our Main dataset is in rough agreement with that reported before [Ashford *et al.*, 1993]. However, the details cannot always be compared directly due to some differences in division into age groups. Ashford *et al.* (1993) used general population data from UN estimates that gave separate percentages for urban and rural population of Syria that were seven years old at the time, and their study population was one of the districts of Aleppo city, which may explain the observed differences. In Çurukova region of Turkey where cutaneous leishmaniasis is also caused by *L. tropica*, the mean age of 1,030 leishmaniasis patients was 22.4 years (in our Main dataset, 17.9 years), and M:F ratio, 40.2:59.8, with more pronounced female predominance [Uzun *et al.*, 2004].

When sex-specific proportional risk ratios (PRR) for each age group were calculated, males of younger ages had slightly higher risk than females, and the

curves crossed in the age group 20-24 years (Figure 2.4). For both sexes the relative risk drops below 1 starting from age group 15-19 years and remains so (linked to puberty?), except for females in age group 50-54 years where a distinct peak to PRR value marginally greater than one from a background about 0.8 is present. The above is true for Main and Treatment registers datasets. This finding may be linked to start of menopause, although in this case it would be expected to have persisted in older age groups, too. A more plausible explanation would be that this peak, together with a smaller one in 60-64-year-old age group in the Main dataset, may be due to "borrowing" case counts from adjacent groups because the former age groups include the values of 50 and 60 years, respectively, and these seem to be stronger attractors than 45 and 55, especially in the Main dataset (Figure 2.2), but this does not explain why similar peaks are not observed among males in the same age groups. In addition, data about possible attractor values in census data are not available, and their presence cannot be ruled out. Available census data show uneven falls of percentages between four age groups, from 40-45 to 60-65 (Figure 2.3), which may support the hypothesis of "borrowing" counts by stronger attractors, 50 and 60 years. Assuming equal strength of attraction in census data and leishmaniasis datasets, our data must be automatically corrected for this bias, and proportional risk ratios must not be affected, so the peaks remain unexplained unless we suppose that females of Aleppo are for some reason more likely to round up their ages compared to general female population of Syria. Looking at age counts in the Main dataset for males and females separately after converting them to percentages would allow calculating ratios of percentages. Figure 2.27 shows that females are at least twice as likely to report ages of 50 and 60 years compared to males, while the same ratio for 55 and 65 years is not much different from one. This may partly explain the peaks, if females in the Main dataset are different from those in Syrian population.

A similar trend in proportional risk ratios, which also fell below one in 15–19 year age group, is published by Ashford *et al.* (1993).

2.5.1.2. Clinical presentation

Statistically significant differences in number of lesions between the three datasets were noted, with fewer patients who present with one lesion in the Treatment dataset than in the Main dataset. About half of the patients presented with one lesion. In Turkey 76% [Uzun *et al.*, 1999] to 80.7% [Uzun *et al.*, 2004] of patients present with one lesion.

There were statistically significant differences in number of lesions between age groups. Older patients present with more lesions on average (Table 2.4). This may also reflect the attitudes of patients who may be more likely to bring their children for treatment than seek treatment for themselves unless "convinced" by higher number of lesions. However, females seem to present with higher number of lesions than males, which is unexpected because the former are supposed to be more concerned about possible cosmetic consequences.

About two-thirds of patients present in the first two months (64.7% in the Main dataset). In contrast, in Turkey the median lesion duration at presentation was five months, and the same proportion, 64.7% reported durations of nine months or less [Dr. S. Uzun, 2005, personal communication]. This may reflect the efficiency of governmental health education programmes in Aleppo or better awareness of the population. Older patients and patients with more lesions have significantly longer lesion duration. This may be confounded by increasing number of lesions with age, which in turn is associated with later presentation. Differences in reported lesion duration between sexes were slightly longer in males but not statistically significant.

It does not appear that more lesions form a greater incentive to seek health care and this is contrary to what would be expected. Differences may be even greater, given that females in these datasets outnumber males and that the

former report marginally shorter lesion duration at presentation. On the other hand, females tend to have more lesions than males, and thus must shift lesion duration in the >2 lesions group (Table 2.10) further down.

This may signify that the patients fall into two distinct groups with regard to health-service seeking behaviour, that can be named 'careful' and 'careless'. The former would seek treatment early in the course of the disease and the latter would postpone their health centre visit until long lesion duration and if numerous lesions convince them to seek diagnosis. The hypothesis would be the latter group are less likely to seek treatment at all if they have few lesions. The fact that the difference is more pronounced among treated patients (the Cards dataset) is in favour of this hypothesis. The 'careful' patients may also care more about returning their cards.

Smear results were positive in 78% of cases, which is close to 82% reported from Turkey [Uzun *et al.*, 2004]. Smear positivity significantly declined with increasing age of patient, which may highlight the differences with Turkey where the average age of the patients is higher. Smear positivity was 68% in lesion durations of less than one month (which probably include a larger proportion of non-leishmanial lesions), then rose to more than 75% in lesions of 1–4 month duration and fell again to below 70% in durations of five months or longer. The decline in parasite numbers with increasing lesion duration was noted since the first description of the parasite by P. Borovsky in 1898 [Hoare, 1938]. There were highly significant differences in smear results between health centres. These may be due to patient or disease characteristics and/or due to differences in staff experience: a 95% positivity rate in one centre can be attributed to false-positives.

In our data the smear positivity declined with increasing age of the patient. A putative explanation for this phenomenon might be that the residents of this endemic region are regularly exposed to infectious sandfly bites which in many cases result in subclinical infections that confer some immunity to the parasite,

that increases with age. If, at older age, they are exposed to an infectious bite that results in manifest disease, their partial immunity due to earlier exposure results in lower parasite burden and decreases the probability of parasite detection by microscopy. This may also explain that overall smear positivity in Aleppo is lower than that reported from an adjacent region of Turkey despite the fact that the study population in Turkey was older and tended to present later [Uzun *et al.*, 2004].

Our data suggest that smear-negative patients with longer lesion duration at presentation are more likely to be treated on the basis of clinical judgement, i.e. despite the negative smear result (Figure 2.16).

2.5.1.3. Health services

More than one-quarter of patients in urban health centres lived in rural Aleppo. This probably reflects unavailability of leishmaniasis diagnosis or treatment in certain villages or the fact that some patients may be unaware these services exist in their local health centre or prefer urban health centres. Rural health centres served almost exclusively rural residents (99.8%). These data come from the period before health distinct policy was enforced (*see* Structure of relevant health services, p. 26). Overall, about forty percent of all patients recorded in 2005 came from rural areas.

2.5.1.4. Response to treatment

Both number of recorded doses and treatment duration were significantly longer in the Patient Cards dataset compared to Treatment registers. Ten percent of the patients recorded in the Treatment registers received only one dose, which may illustrate early drop-out rate: probably, many of the patients who decide to stop treatment do so after the first one or two painful injections, and they would not come to the health centre just to return their card. The same shift to the right is noted between the datasets in distribution of treatment duration.

In the Treatment registers, treatment durations distribute over a wide range, with maximum of 99 weeks, whereas in Patient cards the maximum recorded duration was 50 weeks. This may be due to missing out long courses because the cards of those patients were less likely to be returned to LCC, or may reflect better overall compliance of the patients who returned their cards in the end of the treatment, because they were less likely to miss their appointments.

A clear peak can be distinguished at six doses and five weeks in Treatment registers, probably reflecting a kind of "structuring" of health staff and patients' perceptions concerning the appropriate time to evaluate the treatment effect in the end of the first six-week course. In Patient Cards dataset the mode is twelve doses, corresponding to two courses, *vs.* six in Treatment dataset, which highlights the differences between patients in the two datasets.

In the Patient cards dataset, patients with known cure date differed from those whose cure was not recorded, the latter having significantly longer treatment duration and number of recorded doses. This may be due to difference of health staff attitudes: patients with slower treatment progress are probably less readily rated as cured. The statistical significance of these differences also means the results obtained in the subgroup of patients with known date of cure cannot be reliably generalised neither to all the patients who returned their cards nor to other datasets.

The median treatment duration, number of recorded doses and time to cure was shorter by one (week or dose) in the older, 15+ years, age group, but this difference was statistically significant in the Treatment dataset only ($p<0.0001$, Mann-Whitney U test). This may reflect that younger patients are brought to the centre by adults, and it is more difficult for them to decide to drop out and enforce their decision, but a biological explanation might also be plausible. In a study of American cutaneous leishmaniasis in Peru it was shown that young age was a significant risk factor for treatment failure [Llanos-Cuentas *et al.*, 2008]. If the fact of returning the card can be used as a proxy for completion of

treatment, adults seem less likely to complete their treatment course than children, and if the Patient cards dataset tends to over-represent compliant patients regardless of age, this may explain the lack of statistical significance. However, the Patient cards dataset is smaller, and consequently has less statistical power to detect significant differences.

Females tended to require more doses and longer treatment duration than males, and the differences were significant in Treatment dataset only. On the other hand, the median time to cure in females was two weeks shorter than in males. Although not statistically significant, it is possible that, due to small sample size, the significance could not be detected. If they are significant "in reality" (i.e. were the sample size larger), shorter time to cure in females is contrary to the data on recorded doses and treatment duration, and must be explained by some other factor, e.g. that the females are more compliant, thus more likely to come for treatment appointments on time, and males, if they come at all, miss more appointments on average, probably because they are more likely to have occupational obligations. This is supported by the observation that female-to-male ratio in this subset is nearly 60:40, higher even than in total patient cards sample.

Number of lesions at presentation did not significantly affect the treatment course, although patients with three lesions or more had consistently higher median values for number of recorded doses, treatment duration and time to cure. The only significant difference was the number of recorded doses in the Patient cards dataset (Table 2.17).

The differences in clinical outcomes were significantly different between health centres by Kruskal-Wallis test.

The number of recorded doses and treatment duration, but not time to cure, were significantly lower in patients with longer lesion duration at presentation. This may either reflect the role of acquired immunity or the natural course of the disease.

2.5.2. Notes on data sources

Treatment registers and patient cards were not sufficiently reliable sources of data because, first, both were available only from a minority of health centres (see Differences by health centre, page 79) and, second, because "Cure" field was empty in most instances. This is most probably due to absence of clear definition of cure: staff are taught that cure should not be diagnosed until the patient has finished their follow-up period. During my work in health centres I noticed that at the end of the treatment not all patients are told to come back for follow up after one month; usually they were told to come back if they notice anything unusual with their treated lesion. Nevertheless, in many instances patients finishing their treatment were not recorded as cured in treatment registers; they also were asked to keep their patient cards "just in case". In addition, the field title ("Cure") in treatment registers may be ambiguous: it is not clear if it requires a tick mark, the word "cured" (or "yes"), date of provisionary cure (when patient is judged not to require further treatment) or date of definite cure after completion of the three scheduled follow-up visits.

This may explain both the very low rate of recording date of cure and of returning patient cards. In addition, because these data are not analysed routinely, the staff get no feedback about the missing, incomplete or incorrect data, so have little incentive to improve data quality. In addition, there are currently no facilities for classifying and storing patient cards in health centres. In LCC received patient cards are pooled without classification and stored in cardboard boxes.

Possible biases of data from treatment registers and patient cards make them unsuitable for generalising the results to Aleppo CL population. Yet despite these defects, and partially thanks to them, these differences may provide some insights into underlying causes.

Figure 2.2 shows raw age distribution in datasets. Notable peaks in case counts of ages divisible by 10 and 5 suggest that patients (and/or staff) tend to

round patient's age to the nearest 5 and the nearest 10 years. Another theoretically possible explanation is that patients are not asked about their age, and health centre staff record their own rounded estimates; but in reality staff *do* ask about the patients' age. This illustrates possible inaccuracies in patients' reporting of other information about themselves, e.g. lesion duration. But raw distribution of lesion durations showed only one attractor value: at 12 months.

Other contributing factors may be decrease of smear positivity rate with age (Figure 2.15), which, in turn may be confounded by the fact that older patients tend to report longer lesion duration at presentation, which is also associated with decline in positivity rate (Table 2.10). More sophisticated statistical analysis of available data may reveal relative importance of above-mentioned factors.

Another explanation for younger age of CL patients may lie in differences in treatment-seeking behaviour: younger patients are usually brought to health centres by parents or other related adults; it is the latter who take decision both for seeking health care and treatment on behalf of children. It can be speculated that the same adults may pay less attention to their own minor ailments. This hypothesis cannot be tested using available data, although some indirect evidence may be interpreted in its favour: for example, differences in number of treatment sessions between treatment registers and patient cards, Table 2.14, may indicate that adults are more likely to prematurely stop treatment; on the other hand, mean number of treatment sessions is similar between both age groups in Patient cards dataset (0.1 doses) unlike Treatment registers (where the difference is close to one dose), which may indicate that compliant patients in both age groups need about the same number of doses until discharge; and there are even smaller differences between age groups in the Patient cards dataset with regard to duration of treatment.

Differences between centres in the number of recorded doses (Figure 2.23) are striking, and the ratio between Treatment and Patient cards datasets varies

widely, being largest at LCC, indicating low card return rate. This may be explained, however, by the policy applied in 2005, when diagnosed patients were given the first injection immediately, issued a card and referred to a health centre near their home. And Nayrab health centre is unique because of higher-than-one ratio between mean numbers of doses.

This may indicate differences in practices between health centres or differences in patient characteristics. Differences in time to cure cannot be analysed reliably due to low numbers of cards available from each (maximum 32 cards, Figure 2.25), although it is tempting to note that al-Jalaa health centre requires more than five weeks less than average to cure its patients.

Time to cure was longer by 2-3 weeks than average for patients with lesion duration at presentation of one month, while it was slightly less than average for those who reported lesion durations of 2 or more months at presentation. This may be an indicator of developing immunity in the course of the disease.

Data from three different sources about the 2005 population of cutaneous leishmaniasis patients in Aleppo were compared. Several interesting patterns were noted. But due to incompleteness of this data the significance of observed differences cannot be known. This applies especially to details of clinical course of CL in Aleppo: time to cure cannot be reliably estimated. Patient cards with marked cure date might provide the best estimate but the available sample is clearly biased. This would justify a more robust data collection and analysis.

2.6. Tables

Table 2.1. Age, mean values and quartiles: Syrian population (census 2004), and main dataset, treatment registers, patient cards and patients with known cure date (2005).

	Census	Main	Treatment	Patient cards	Known cure date
Mean	unknown	17.94	16.48	16.62	17.42
25th percentile	9	6	5	6	7
Median	19	13	11	12	12
75th percentile	33	25	23	22	23

Table 2.2. Sex distribution in Syria and Aleppo according to 2004 census, and in the datasets of 2005 leishmaniasis patients.

	Syria	Aleppo	Main	Treatment	Patient cards	Known cure date
Males	51.1	51.5	47.7	48.9	43.2	40.7
Females	48.9	48.5	52.3	51.1	56.8	59.3

Table 2.3. Distribution of patients by place of residence between urban and rural health centres (Main dataset).

	Patients residence		
Centre location	**Urban**	**Rural**	**Total**
Urban	8587 (72.9%)	3200 (27.1%)	11787 (100%)
Rural	4 (0.2%)	2577 (99.8%)	2581 (100%)
Total	8591 (59.8%)	5777 (40.2%)	14368 (100%)

Table 2.4. Mean and median number of lesions in two age groups in the three datasets.

Dataset	**Mean**		**Median**		**Mann-Whitney U**
Age group	*0–14*	*15+*	*0–14*	*15+*	
Main	1.9	2.5	1	2	$p<0.0001$
Treatment	2.0	2.8	1	2	$p<0.0001$
Cards	1.9	2.2	1	2	$p = 0.015$

Table 2.5. Mean and median number of lesions by sex in the three datasets.

Dataset	Mean		Median		Mann-Whitney U
Sex	*M*	*F*	*M*	*F*	
Main	2.18	2.31	1	1	p=0.0004
Treatment	2.26	2.39	1	2	p=0.251
Cards	1.93	2.08	1	1	p=0.045

Table 2.6. Regression analysis of factors that affect number of lesions (one or two *vs.* three or more).

		Univariate analysis				Multivariate analysis			
			CL				CL		
Variables	**Category***	**OR**	**lower**	**upper**	**p**	**OR**	**lower**	**upper**	**p**
Sex	*Female*	1.180	1.094	1.273	<0.0001	1.140	1.056	1.231	0.001
Age group	*15+ years*	1.678	1.556	1.810	<0.0001	1.677	1.553	1.810	<0.0001
Lesion noted	*Dec – July*	0.611	0.565	0.659	<0.0001	0.608	0.562	0.657	<0.0001

* The reference categories were assigned as follows: sex, male; age group, 0–14 years; calculated month of lesion appearance ("lesion noted"): August to November. OR, odds ratio.

OR, odds ratio; CL, 95% confidence limits.

Table 2.7. Lesion locations by sex. *One lesion* section: patients with one lesion. The *One location* section: patients whose lesions were confined to one location, whether solitary or multiple. The percentages add up to less than 100% for each sex because other locations are excluded. *Any lesions on face* section shows the data for the complete dataset, comparing patients who had any lesions on face with those whose lesions were in other locations.

	Male	Female	p (χ^2)
Lesion location	**One lesion** *(n=7,650)*		**1 lesion**
Face	44.8%	41.9%	0.01
Hand	34.3%	39.6%	<0.0001
Leg	16.3%	16.7%	0.652
% of total	95.4%	98.2%	
	One location *(n=12,176)*		
Face	44.7%	38.0%	<0.0001
Hand	35.9%	45.0%	<0.0001
Leg	16.1%	15.6%	0.481
% of total	96.7%	98.6%	
	Any lesions on face *(n=14,368)*		
Face	46.8%	40.4%	<0.0001

Table 2.8. Lesion locations by age group. The *One lesion* section: patients with one lesion. The *One location* section: patients whose lesions were confined to one location, whether solitary or multiple. The percentages add up to less than 100% for each age group because other locations are excluded. *Any lesions on face* section shows the data for the complete dataset, comparing patients who had any lesions on face with those whose lesions were in other locations.

Age group	0-14 years	15+ years	p (χ^2)
Lesion location	**One lesion** *(n=7,650)*		
Face	55.9%	24.8%	<0.0001
Hand	25.0%	54.8%	<0.0001
Leg	15.3%	18.3%	0.0005
% of total	96.2%	97.9%	
	One location *(n=12,176)*		
Face	57.9%	19.6%	<0.0001
Hand	24.3%	61.8%	<0.0001
Leg	14.9%	17.1%	0.0008
% of total	97.1%	98.5%	
	Any lesions on face *(n=14,366)*		
Face	59.4%	23.7%	<0.0001

Table 2.9. Regression analysis of factors that affect lesion location (face *vs.* non-face).

		Univariate analysis				Multivariate analysis			
			CL				CL		
Variables	**Category***	**OR**	**lower**	**upper**	**p**	**OR**	**lower**	**upper**	**p**
Sex	*Female*	0.769	0.720	0.822	<0.0001	0.812	0.756	0.872	<0.0001
Age group	*15+ years*	0.213	0.198	0.229	<0.0001	0.217	0.202	0.234	<0.0001
Lesion noted	*May – Aug*	0.579	0.527	0.636	<0.0001	0.609	0.552	0.673	<0.0001
	Sep – Dec	1.131	1.050	1.218	0.001	1.135	1.048	1.229	0.002

* The reference categories were assigned as follows: sex, male; age group, 0–14 years; calculated month of lesion appearance ("Lesion noted"): January to March.

OR, odds ratio; CL, 95% confidence limits.

Table 2.10. Mean lesion duration at presentation (in months) in the subgroups by age, sex and number of lesions. The median values are 2 months in all the subgroups.

		Mean lesion duration at presentation *(months)*			
		Main	p value*	Cards	p value*
Age	**0–14**	2.60	<0.0001	2.51	0.025
	15+	3.22		3.01	
Sex	**M**	2.91	0.126	2.93	0.305
	F	2.85		2.54	
Number of lesions	**1 or 2**	2.85	<0.0001	2.54	0.001
	3+	2.94		3.28	

* Mann-Whitney U test.

Table 2.11. Mean reported lesion duration at presentation (months) by lesion location in patients with solitary or multiple lesions in one of the three most common locations, compared to those with lesions in other locations. The last two columns compare patients with any lesions on face with those who had no lesions on face. The median values were equal to 2 months in all cells, p<0.0001 (Mann-Whitney test) for all comparisons. *Yes* and *No* refer to the lesion locations in rows.

	One lesion		One lesion or more		Any lesion(s) on face	
Lesion location	*yes*	*no*	*yes*	*no*	*yes*	*no*
Face	2.51	3.15	2.51	3.11	2.56	3.12
Hand	3.11	2.74	3.07	2.73		
Leg	3.21	2.81	3.19	2.81		

Table 2.12. Smear results in the Main dataset and Patient cards.

Dataset	Smear result	
	Pos	Neg
Main	78.0%	22.0%
Patient Cards	90.6%	9.4%

Table 2.13. Odds ratios (OR) of smear positivity in five age groups. The youngest age group serves as the reference category.

		Univariate analysis				Multivariate analysis			
			CL				CL		
Age group*	n	OR	lower	upper	p	OR	lower	upper	p
0–9	5446	1.000			<0.0001	1.000			<0.0001
10–19	4043	0.752	0.679	0.832	<0.0001	0.749	0.677	0.830	<0.0001
20–29	1991	0.666	0.588	0.753	<0.0001	0.676	0.597	0.766	<0.0001
30–39	1202	0.656	0.565	0.761	<0.0001	0.657	0.566	0.763	<0.0001
40+	1684	0.505	0.445	0.572	<0.0001	0.503	0.444	0.571	<0.0001

* The age group 0–9 years is the reference category, the corresponding p value is for the overall regression model. The multivariate analysis model included lesion duration at presentation and health centre location (urban *vs.* rural).

OR, odds ratio; CL, 95% confidence limits.

Table 2.14. Comparison between recorded doses, treatment duration and time to cure in the Treatment register (n=2145) and Patients cards (n=498) datasets. Treatment duration and time to cure are in weeks. Time-to-cure data were available for 189 patients.

	Recorded doses		Treatment duration		Time to cure
Dataset	*Treatment*	*Cards*	*Treatment*	*Cards*	*Cards*
Mean	6.80	11.61	10.02	15.40	19.58
Percentile:					
10	1	5	0	5	9
25	3	7	3	8	12
median	6	10	7	12	18
75	9	15	14	23	27
90	12	20	22	30	32
Maximum	24	40	99	50	50

Table 2.15. Median values by age group of the number of recorded doses and duration of treatment in Treatment and Patient Cards datasets, and time to cure in the Patient Cards dataset. Treatment duration and time to cure are in weeks.

		Age group		p*
		0–14	15+	
Recorded doses	**Treatment**	7	6	<0.0001
	Cards	10	11	0.667
Treatment duration	**Treatment**	8	7	<0.0001
	Cards	12	12	0.696
Time to cure	**Cards**	19	17	0.198

* Mann-Whitney U test

Table 2.16. Differences between sexes in the number of recorded doses, treatment duration and time to cure.

		Sex		p*
		M	F	
Recorded doses	Treatment	6	7	0.0002
	Cards	10	10	0.919
Treatment duration	Treatment	7	8	<0.0001
	Cards	11.5	13	0.690
Time to cure	Cards	19	17	0.522

* Mann-Whitney U test

Table 2.17. Differences in recorded doses, treatment duration and time to cure according to number of lesions at presentation.

		Lesions at presentation		p*
		1 or 2	3 or more	
Recorded doses	Treatment	6	7	0.333
	Cards	10	12	0.026
Treatment duration	Treatment	7	8	0.778
	Cards	12	14.5	0.180
Time to cure	Cards	17	19	0.290

* Mann-Whitney U test

2.7. Figures

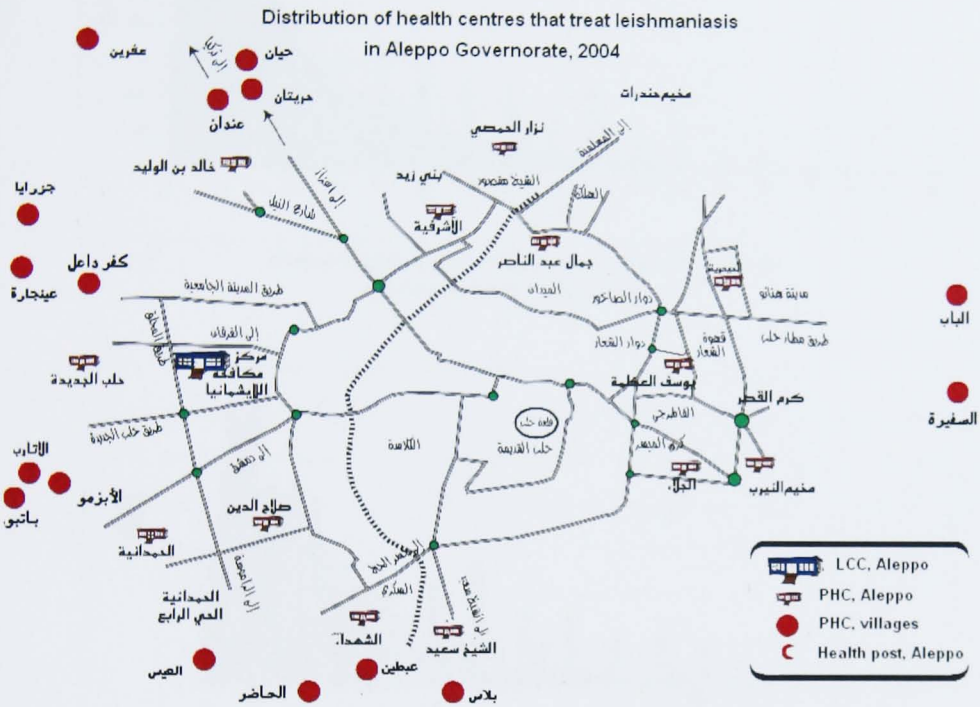


Figure 2.1. Schematic map of health centres that treat leishmaniasis patients in Aleppo city and coutryside as of 2004. Locations of village centres are not to scale.

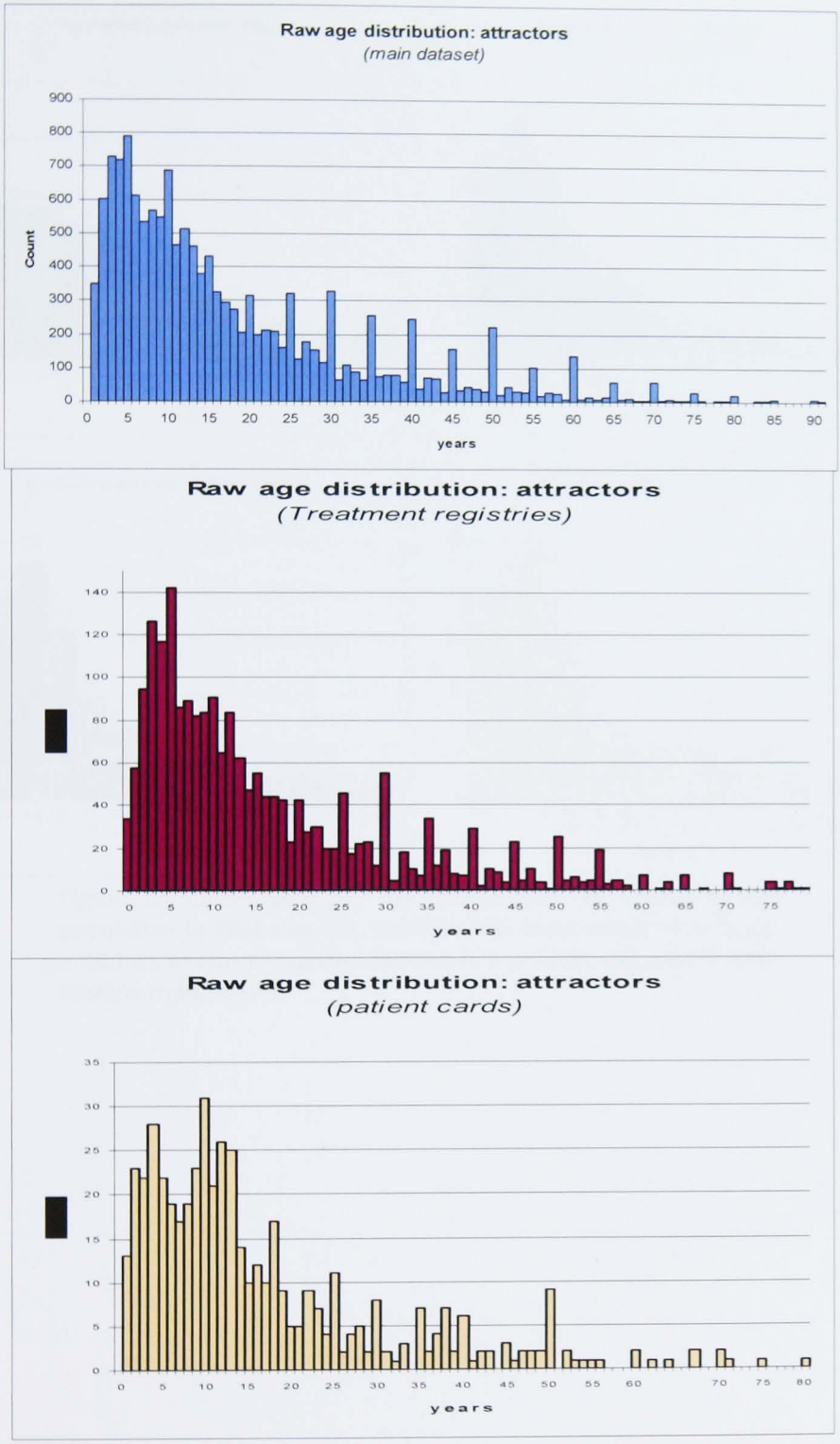


Figure 2.2. Raw age distribution of patients from three datasets shows clear peaks of counts at ages that divide by five, especially in the two larger datasets (attractor values).

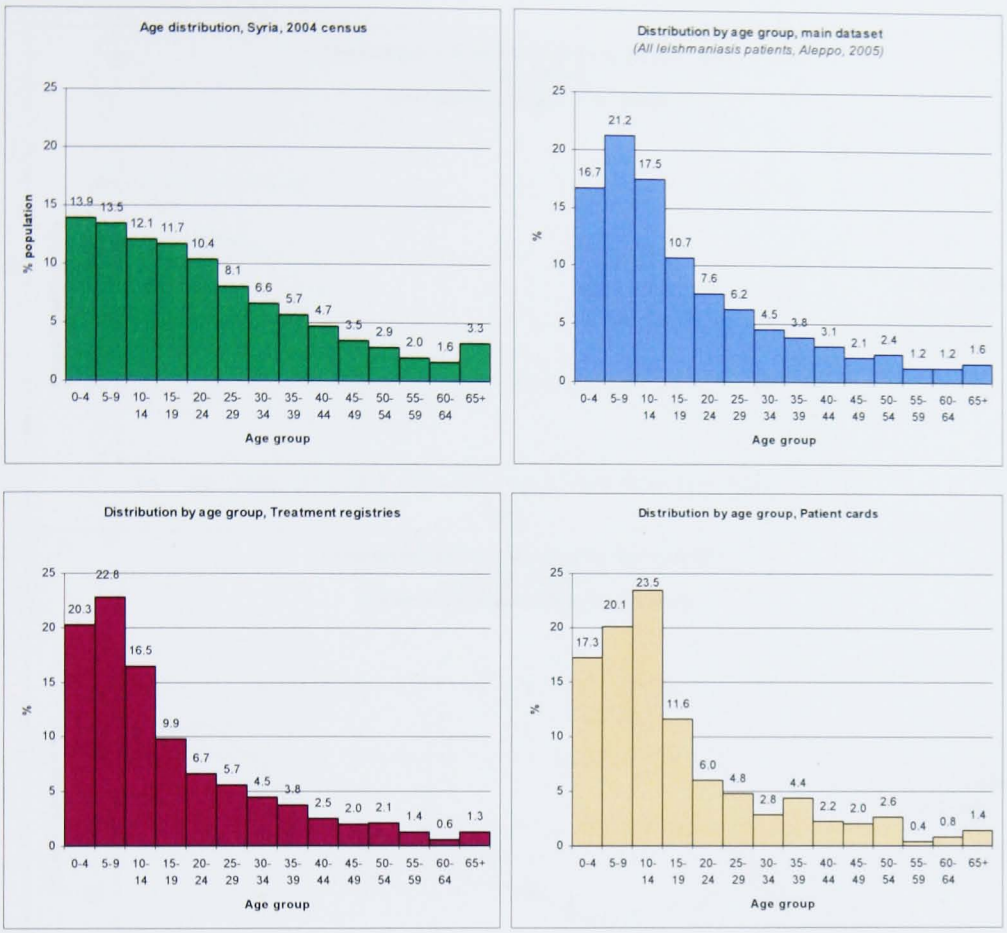


Figure 2.3. Distribution by age groups used in census: Syrian population in 2004 (top left, n=17,921,000), main dataset (top right, n=14,368), treatment registers (bottom left, n=2,138) and patient cards (bottom right, n=498).

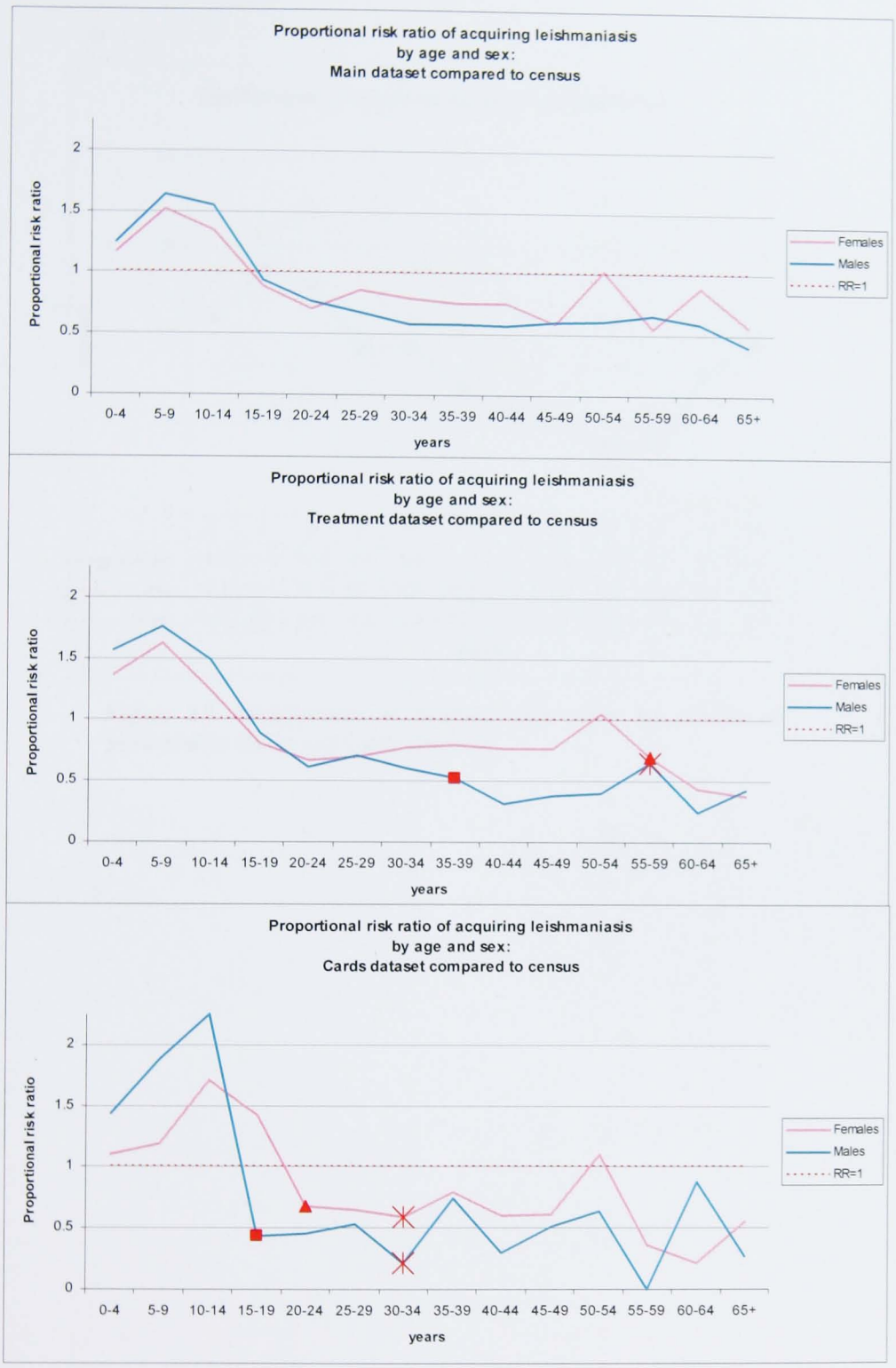
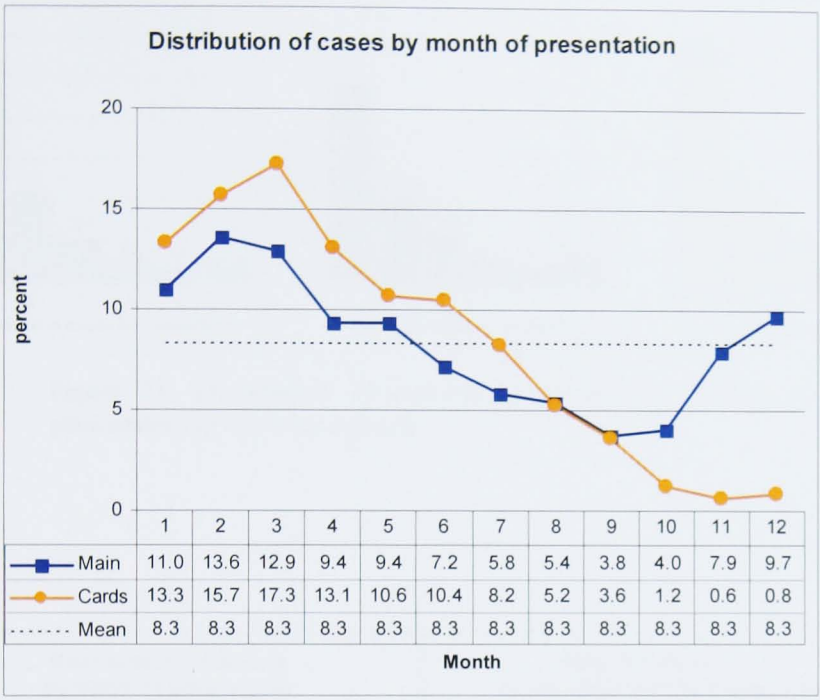


Figure 2.4. Proportional risk ratio of leishmaniasis by sex and age group compared to census. Squares (males) or triangles (females) indicate patient counts ≤20 for each sex, and asterisks, total patient count ≤30 in the marked age group and all age groups that follow.



	1	2	3	4	5	6	7	8	9	10	11	12
Main	11.0	13.6	12.9	9.4	9.4	7.2	5.8	5.4	3.8	4.0	7.9	9.7
Cards	13.3	15.7	17.3	13.1	10.6	10.4	8.2	5.2	3.6	1.2	0.6	0.8
Mean	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3	8.3

Figure 2.5. Distribution of leishmaniasis cases by month of presentation (Main and Cards datasets).

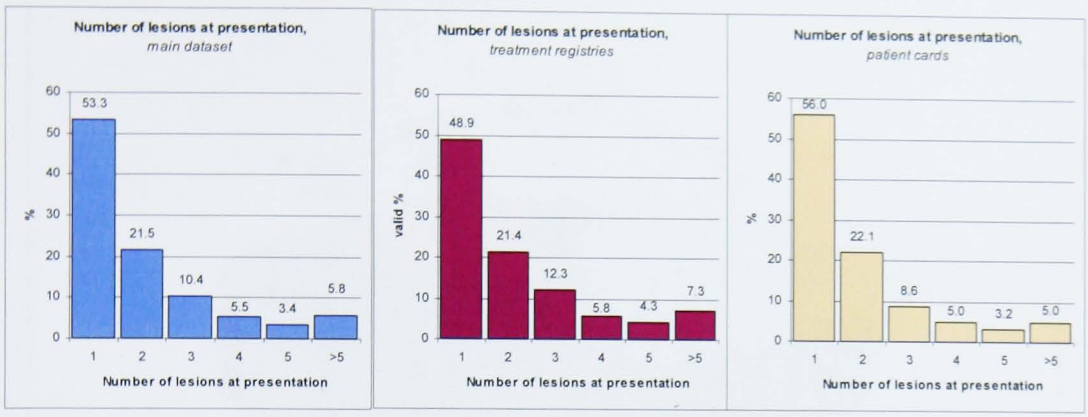


Figure 2.6. Distribution of patients by number of lesions at presentation in the three datasets.

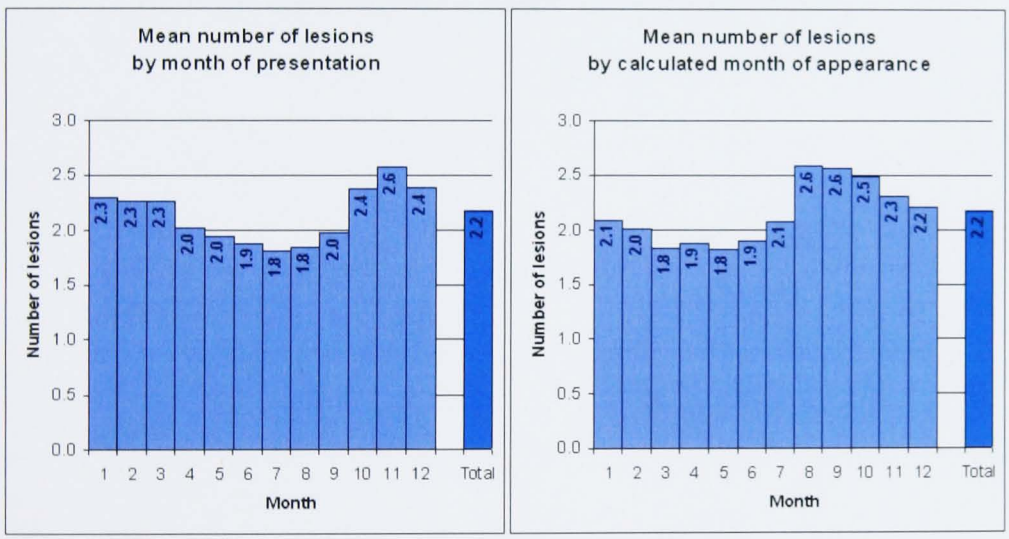


Figure 2.7. Number of lesions by month of presentation and calculated month of appearance.

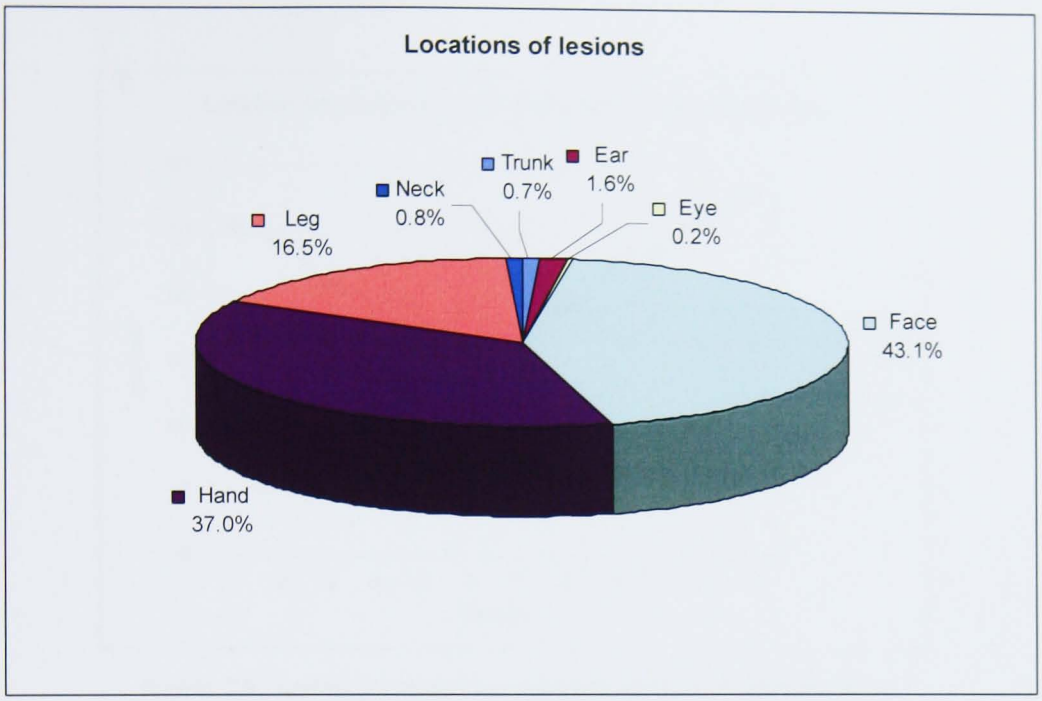


Figure 2.8. Lesion locations in patients with one lesion (n=7650).

Figure 2.9. Lesion locations by reported month of appearance in patients with one lesion who reported lesion duration of less than 12 months (n=7,392; total in any month >300). Figure 2.10. Lesion locations by reported month of appearance in patients with one lesion who reported lesion duration of less than 12 months (n=7,392; total in any month >300).

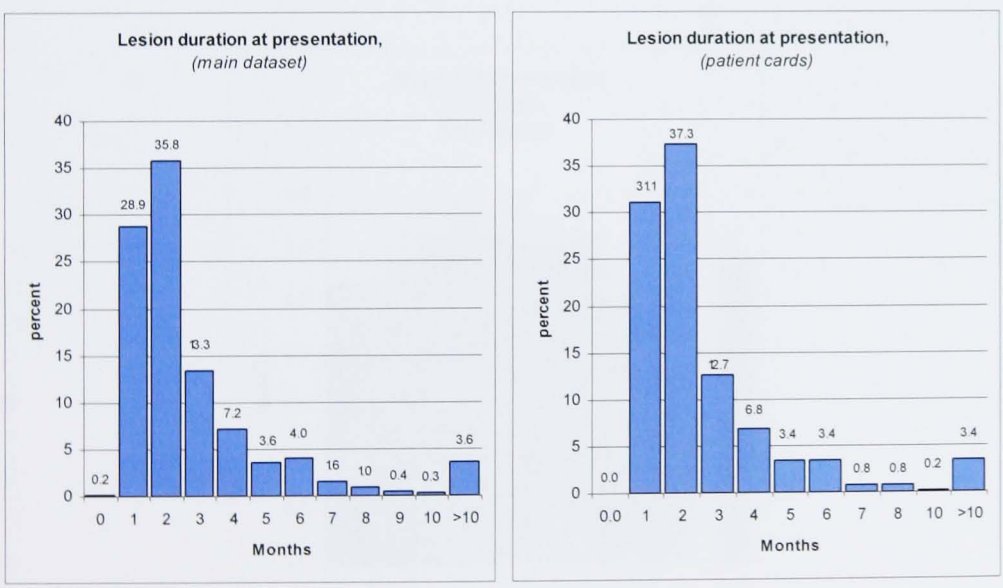


Figure 2.11. Reported lesion duration at presentation.

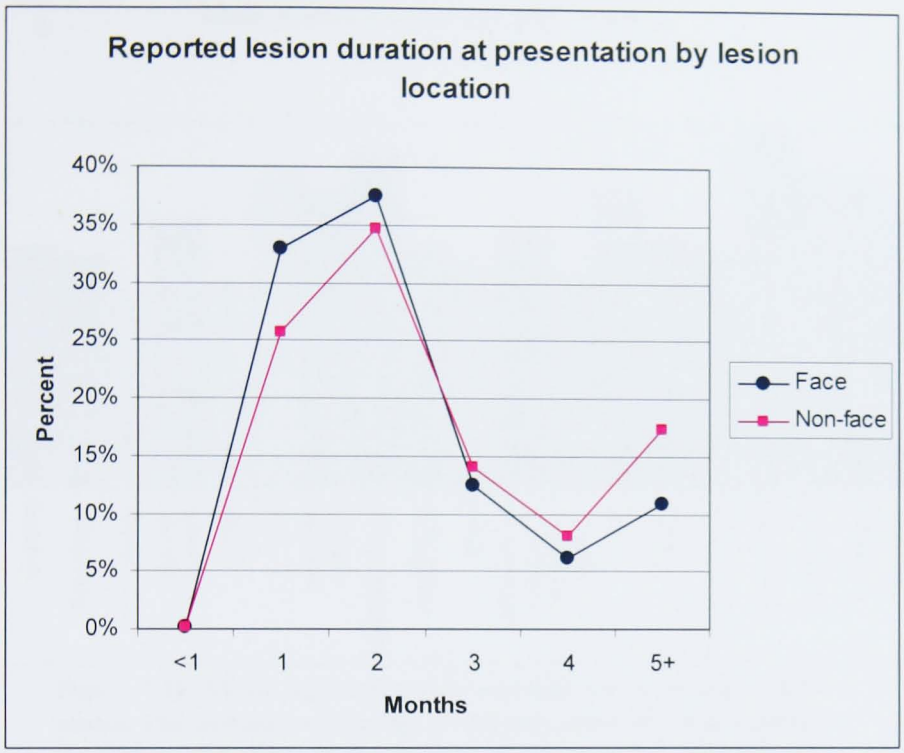


Figure 2.12. Relationship between lesion location and reported lesion duration. Patients who had any lesion(s) on face (circles) vs. those who had no lesions on face (squares) (n=14,368).

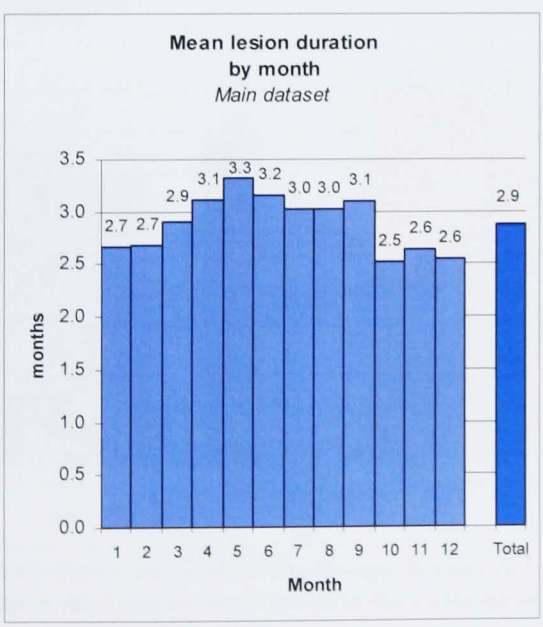


Figure 2.13. Reported lesion duration by month of presentation.

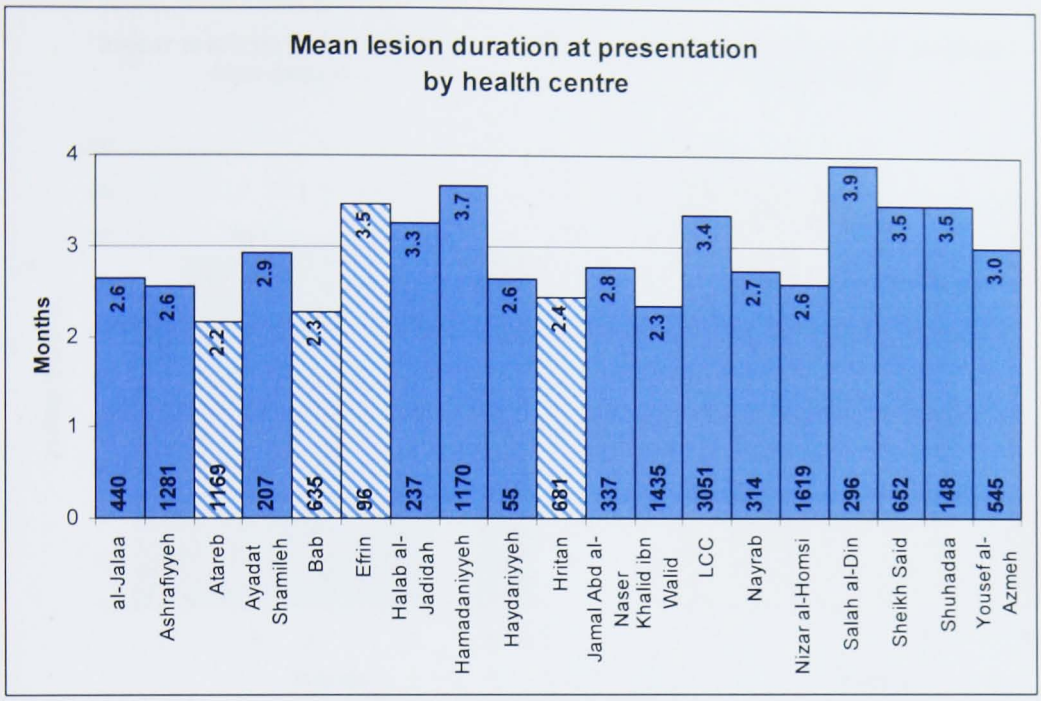


Figure 2.14. Mean reported lesion duration by reporting health centre. The numbers at the top of the bars show the mean lesion durations, and those at the bottom of the bars the numbers of reported cases. Diagonal pattern indicates the four rural health centres.

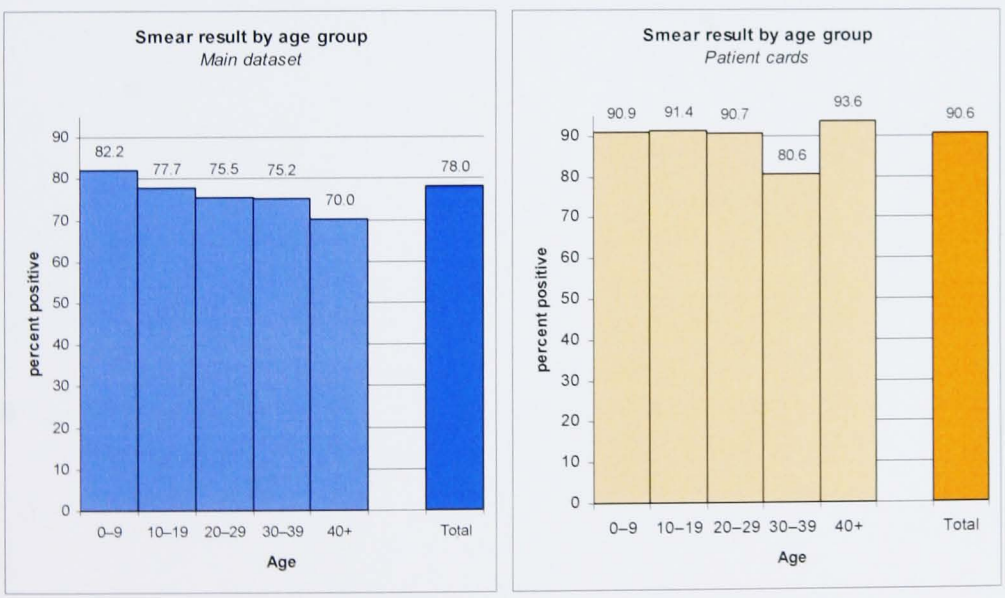


Figure 2.15. Smear result positivity by age. A clear decline with age is evident in the Main dataset but not in the Cards dataset.

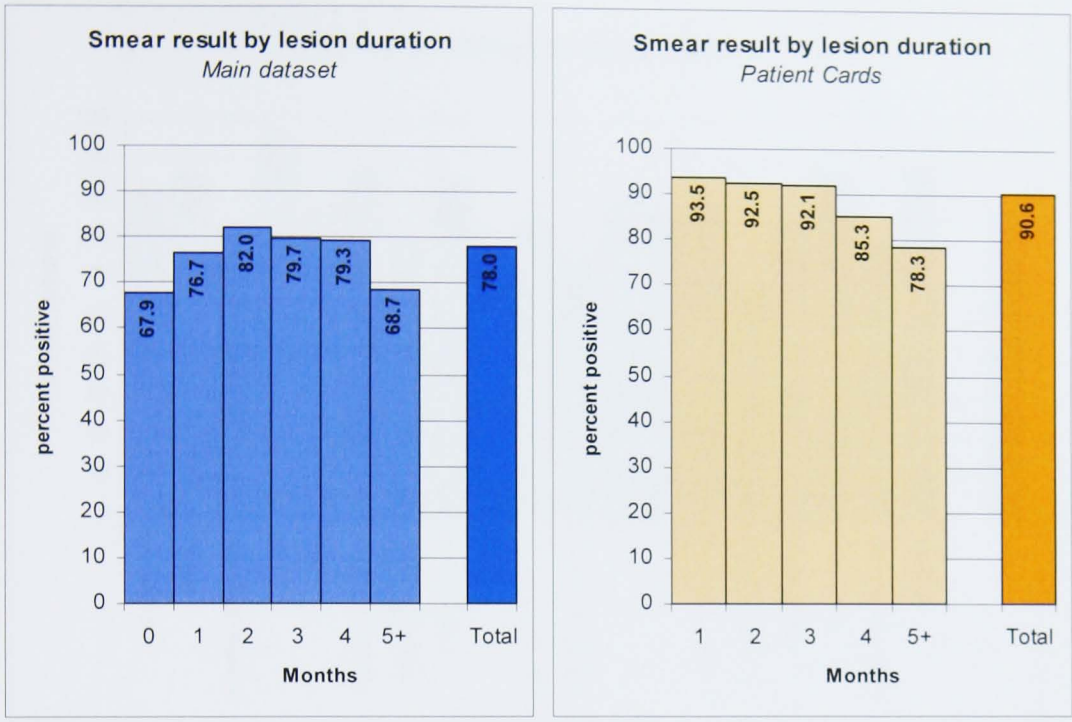


Figure 2.16. Smear result positivity by reported lesion duration.

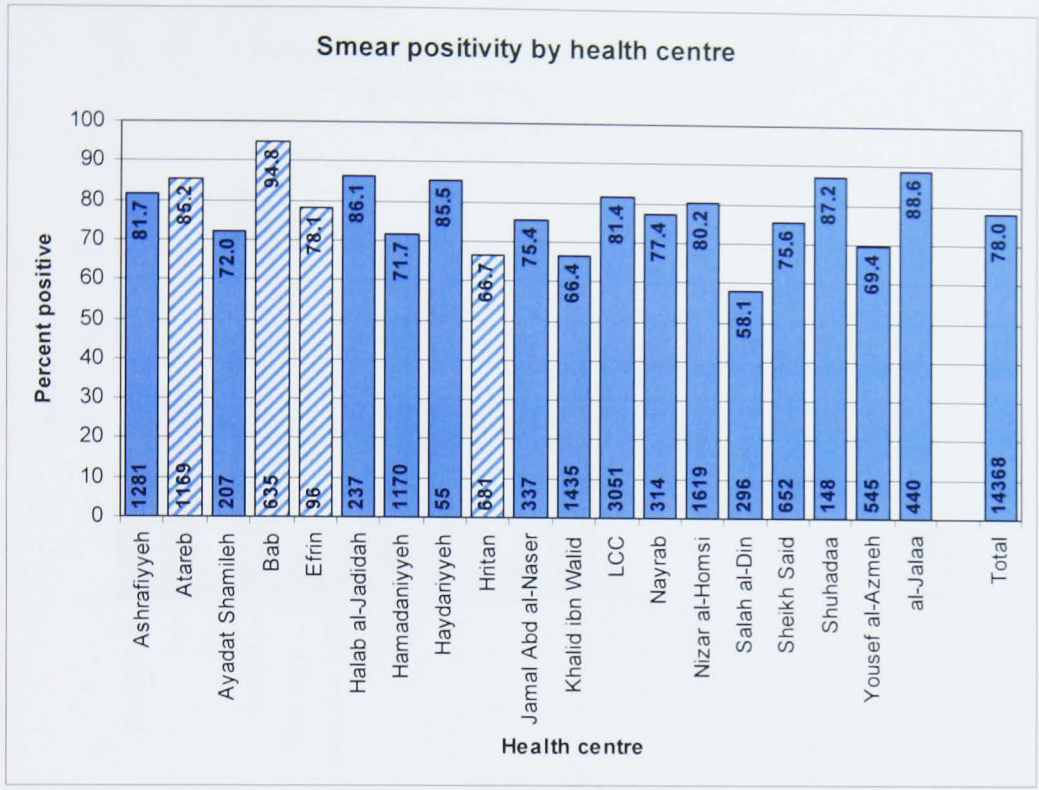


Figure 2.17. Smear positivity by health centre. The numbers at the top of the bars are percentages of positive smear results. The numbers at the bottom of the bar are those of reported patients. Rural health centres are indicated with diagonal pattern.

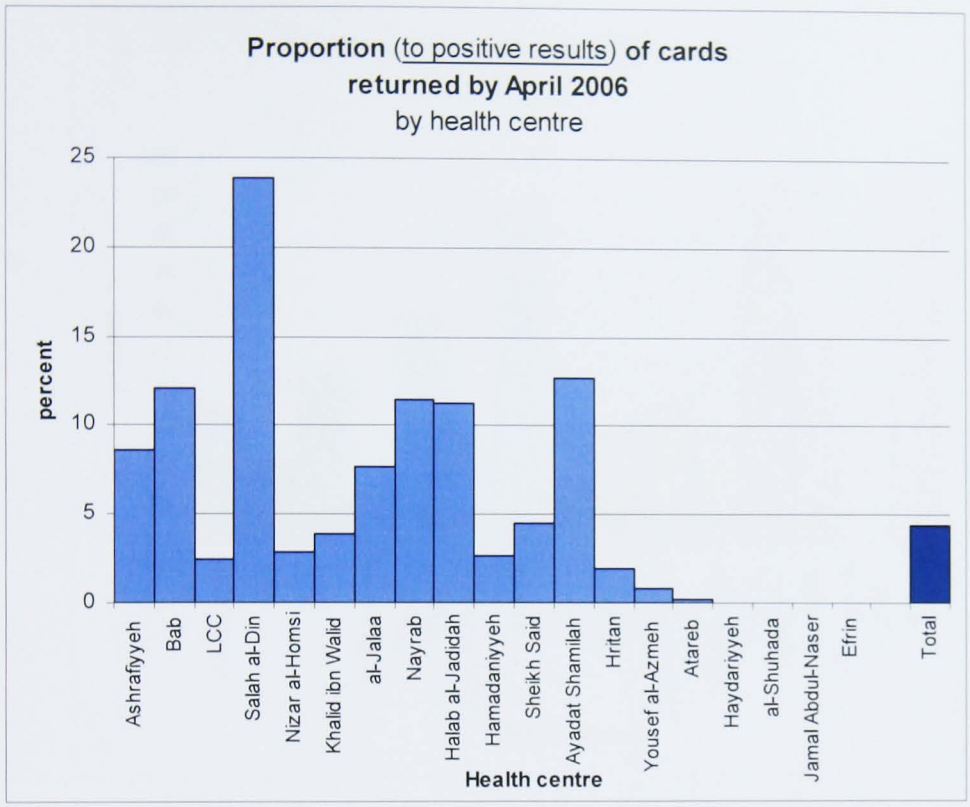


Figure 2.18. Proportion of cards of patients who started treatment in 2005 that were available from each health centre by April 2006. The number of smear-positive patients recorded in each of these centres was taken as denominator.

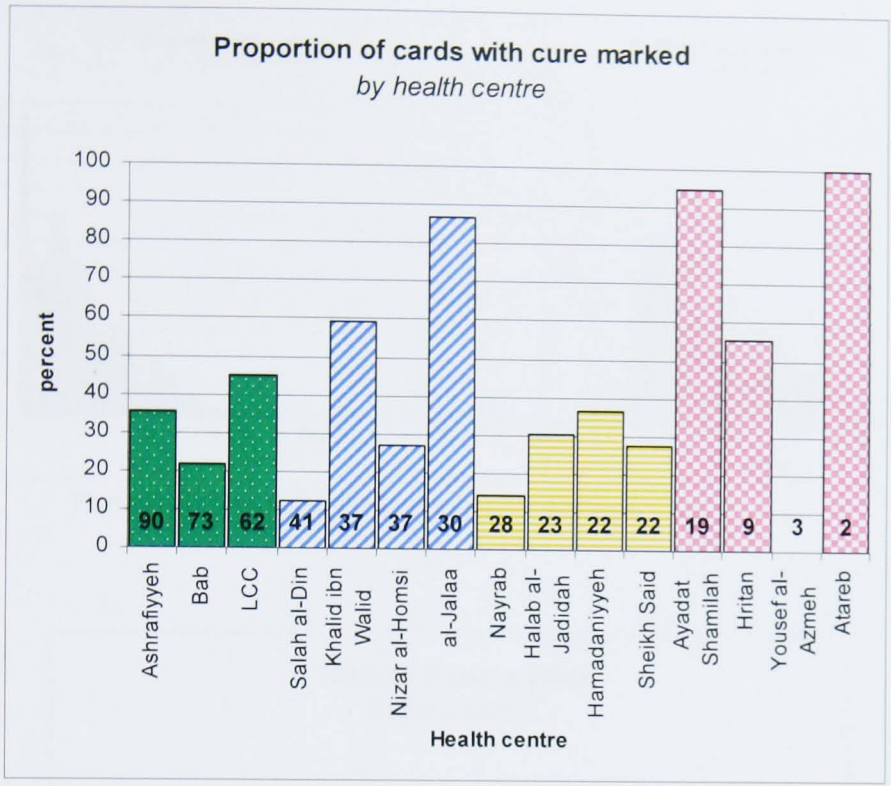


Figure 2.19. Percentage of cards from each health centre with date of cure recorded. Numbers of available patient cards are indicated by colour and pattern — green, dotted: ≥60; blue, diagonal: 30–59; yellow, horizontal: 20–29; and pink, checkerboard: <20. The numbers at the base are those of cards available from each centre which were used as denominators for percentages.

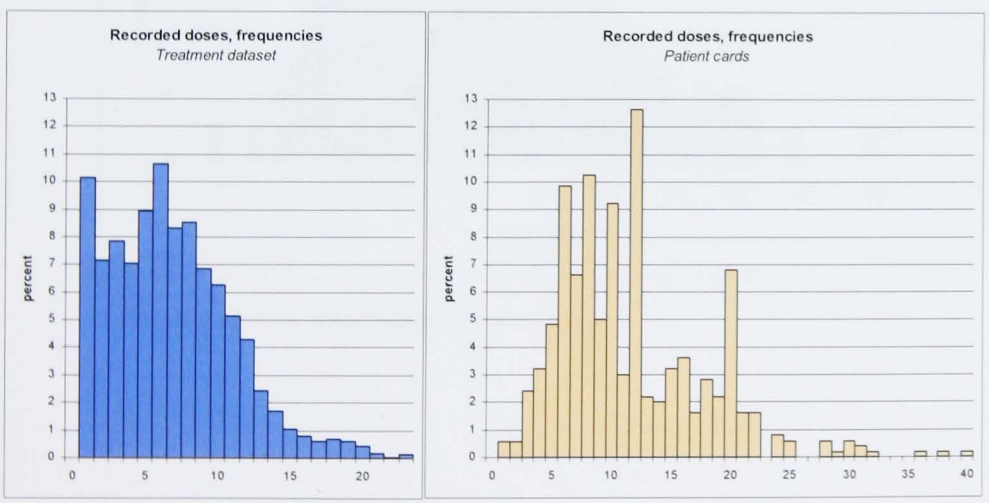


Figure 2.20. Raw distribution of recorded doses (percent).

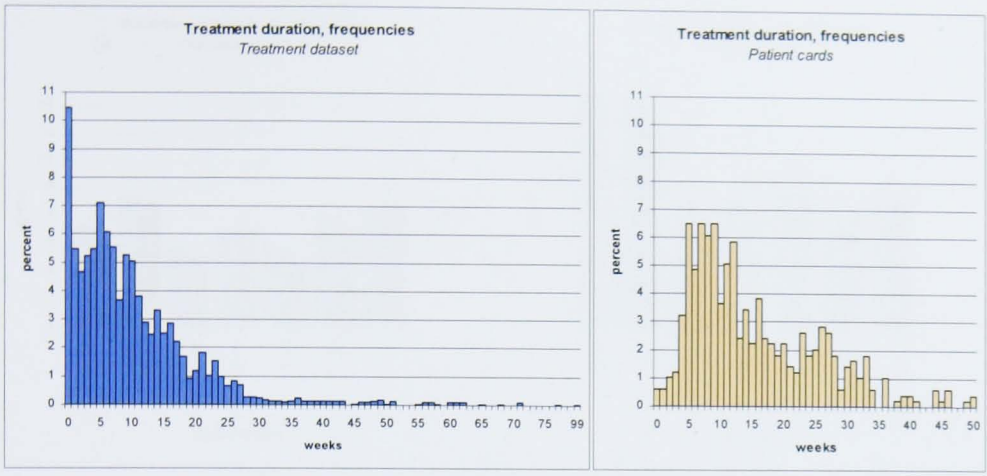


Figure 2.21. Raw distribution of treatment duration.

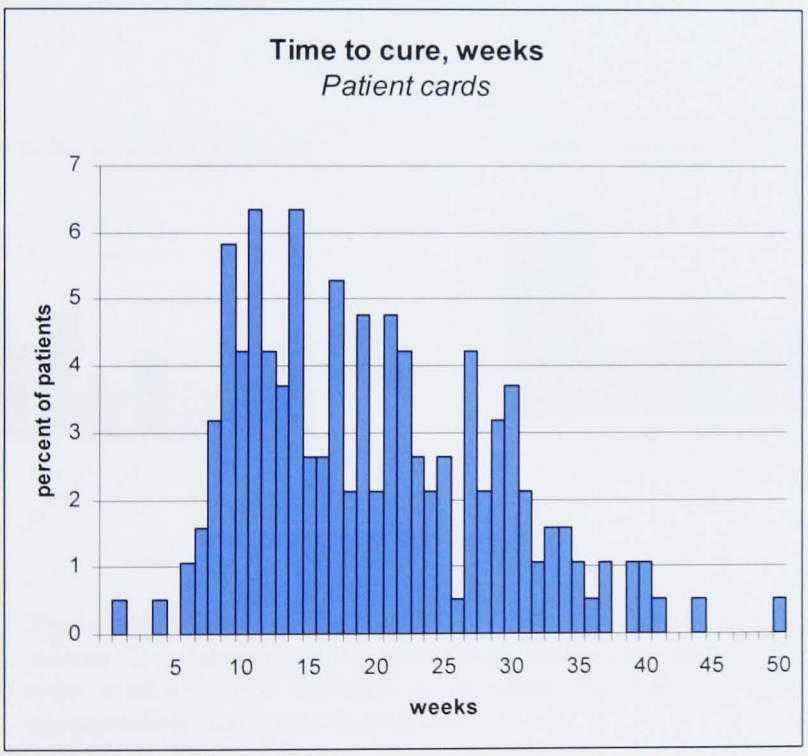


Figure 2.22. Raw distribution of time to cure.

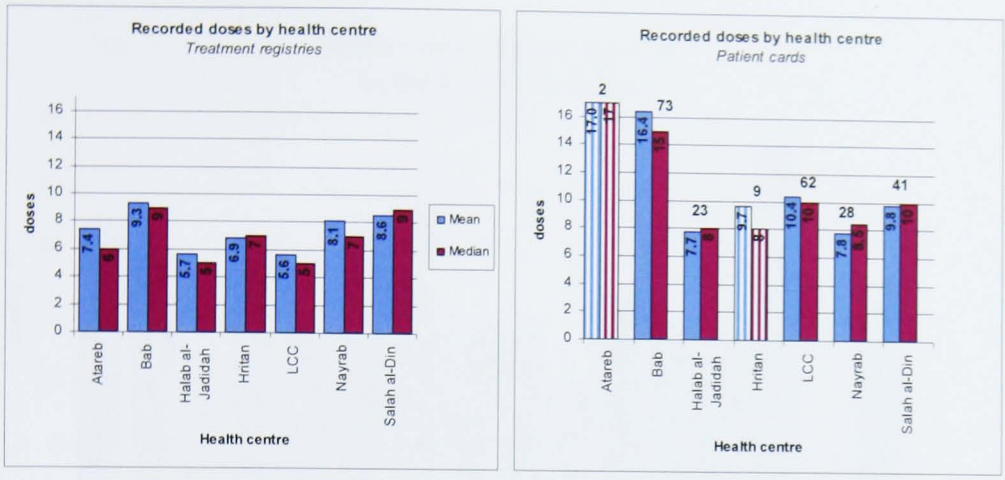


Figure 2.23. Differences in number of recorded doses by health centre. The numbers above the bars are those of patient cards available from each centre, and the striped bars indicate the health centres with number of available cards <10.

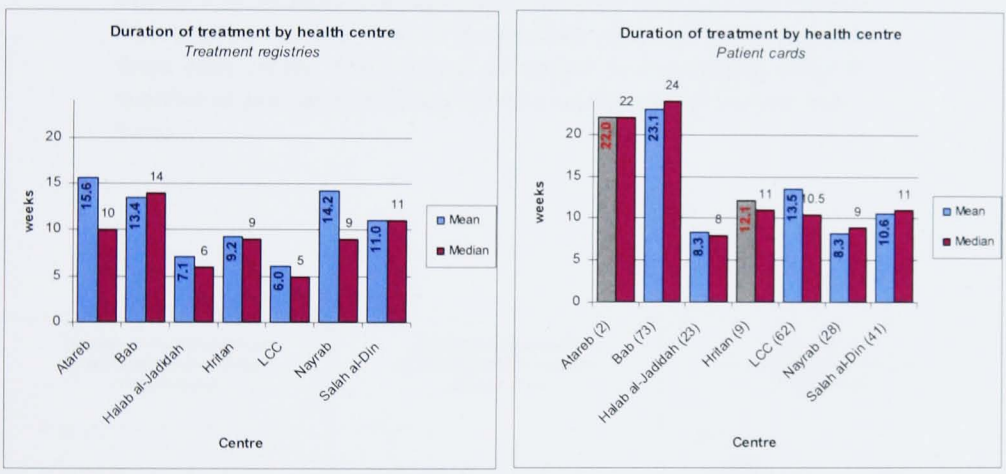


Figure 2.24. Differences in duration of treatment between health centres. Grey bars indicate the centres from which <10 patient cards were available. The numbers in or above the bars are the corresponding mean or median values.

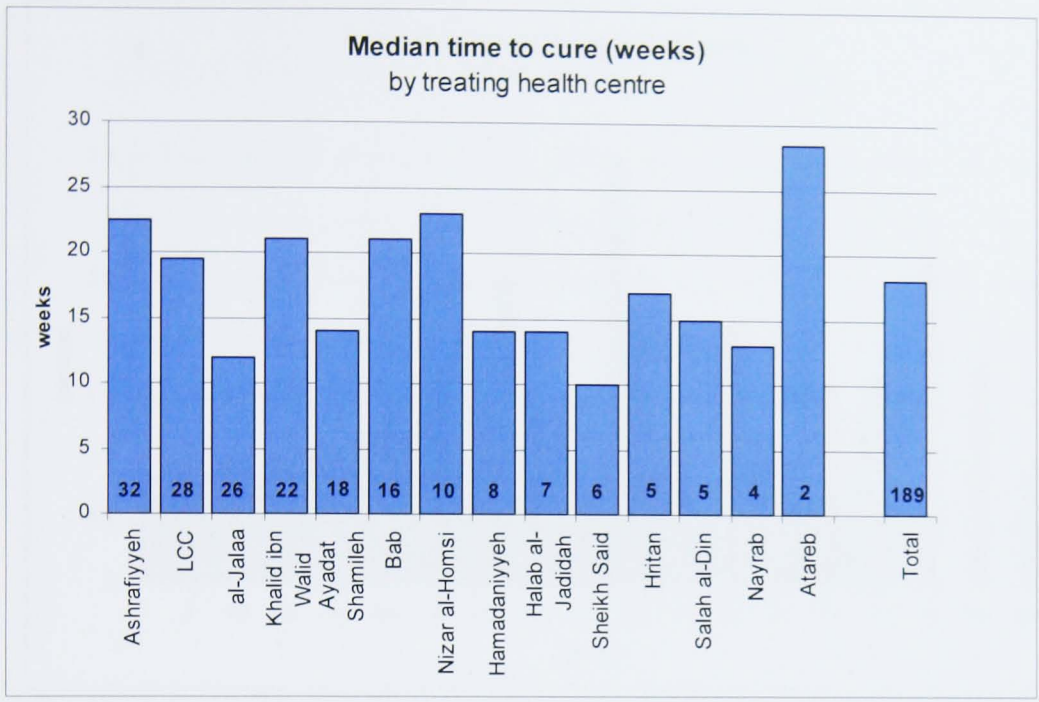


Figure 2.25. Median time to cure (weeks) by treating health centre. The numbers at the base of the bars are those of cards available from each centre. The centres are sorted in descending order by number of available cards (indicated near the base of corresponding bars).

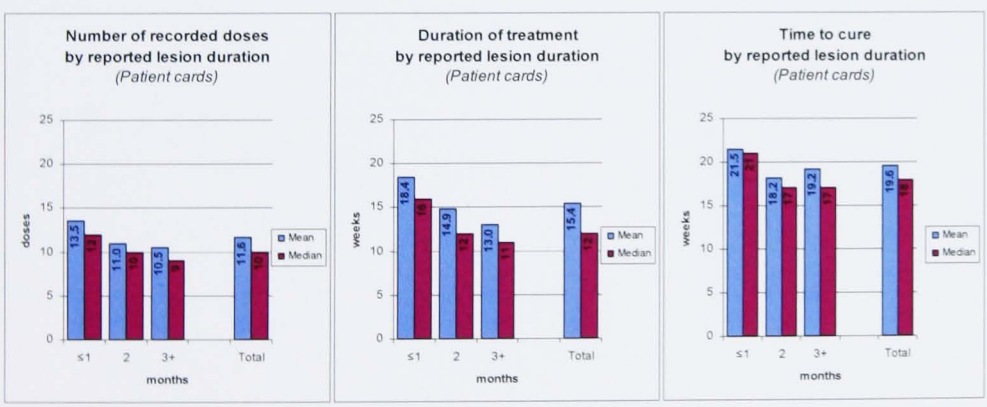


Figure 2.26. Number of recorded doses, duration of treatment and time to cure by reported lesion duration at presentation. Patient cards dataset.

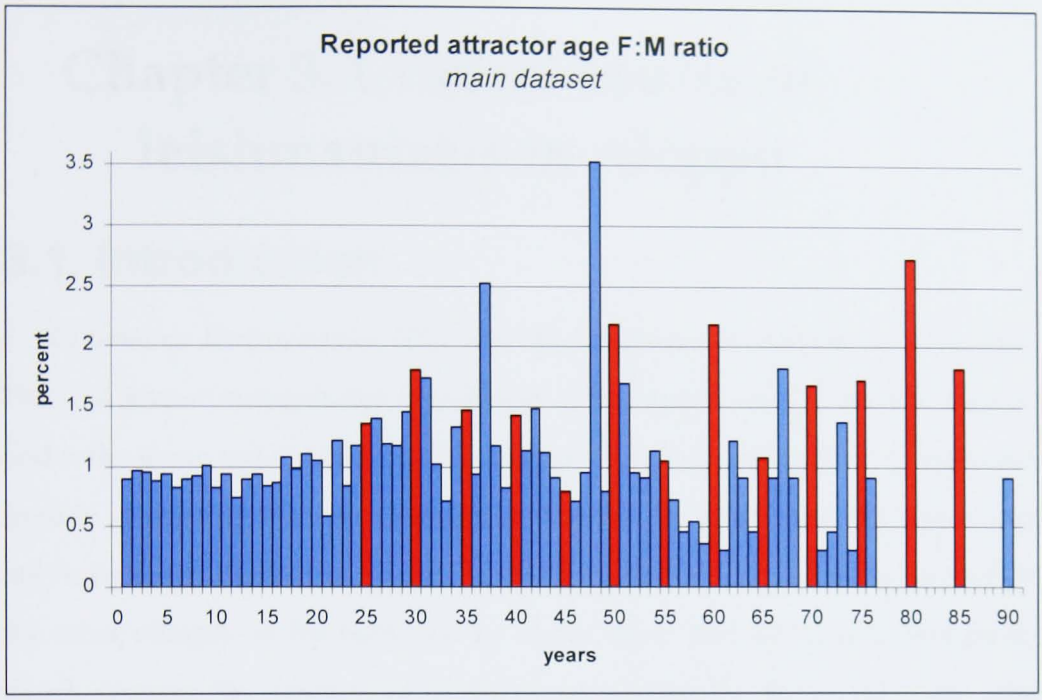


Figure 2.27. Female-to-male ratio of reported age (Main dataset). Attractors are marked red. Total number of patients in attractor age groups falls below 60 starting from 65-year-old age group and below 30 starting from 75-year-old group.

Chapter 3. Clinical course of leishmaniasis in Aleppo

3.1. Introduction

Cutaneous leishmaniasis (CL) has been endemic in Aleppo for centuries. The first known recognisable description of the disease and its clinical course, under the name *mal d'Aleppo*, was published in 1756 by Alexander Russell who lived in Aleppo for thirteen years [Russell, 1856]. He noted that the disease was not restricted to the city of Aleppo, "...being almost as common at *Antab*, and all the other villages on the banks of the rivers *Sejour* and *Coick*, as at this place; which favours the opinion of it being occasioned by the water." He also mentioned that "[t]he natives call it *Habbt il senne*, or *Botch of a year*, from the supposed time of its duration." This vernacular name is still common until present in Aleppo; outside Aleppo in Syria it is known as "*habbet Halab*", or the sore of Aleppo [Elgood, 1934].

In 1929, T. Canaan wrote: "The disease in Aleppo is so common as to be proverbial amongst the Arabs of Syria and Palestine" [Canaan, 1929]. Several years later the same (about Syria and Lebanon) was mentioned by Hovnanian [Hovnanian *et al.*, 1937] who described an outbreak of cutaneous leishmaniasis in Aleppo in a community of refugees with disease prevalence of 43%.

Cutaneous leishmaniasis in Aleppo is caused by *Leishmania tropica*, and the putative vector is *Phlebotomus sergenti* [Ashford *et al.*, 1993]. *Leishmania tropica* is generally believed to be anthroponotic [Desjeux, 2001]. As reviewed in Chapter 1 and Table 1.3, *L. tropica* infection tends to run a longer course compared to *L. major*, and is more likely to cause chronic disease or relapse after apparent cure [Herwaldt, 1999]. No other causative agents nor leishmaniasis forms were identified in Aleppo Governorate to date, although a few cases of

infantile visceral leishmaniasis are recorded in small foci in adjacent coastal (Lattakia and Tartous) and Idlib governorates. The forms encountered in Syria are also reviewed in more detail in Chapter 1.

It is believed that insecticide spraying as part of malaria control efforts in 1950s lead to a concomitant fall in leishmaniasis cases in Syria [Jalouk *et al.*, 2007]. During the following years only a few hundred cases were reported from Aleppo, but from 1985 there was a considerable rise in the reported incidence of leishmaniasis that could not be fully attributed to improved case detection and reporting [Ashford *et al.*, 1993]. Three peaks over 8,000 cases occurred in 1991, 1994 and 2001, and more than 11,000 cases in 2003–2005, despite two campaigns of insecticide spraying every year.

This huge number of cases in a large city could not be managed by one specialised control centre. For this reason, leishmaniasis diagnosis and control facilities were introduced into an increasing number of primary health care (PHC) centres. Due to logistic constrains, not all of these have laboratory diagnostic facilities but a member of staff was trained to administer intralesional injections to the patients from the catchment area of the PHC centre, who were diagnosed elsewhere: either at the main Leishmaniasis Control Centre (LCC) or in a nearby PHC centre where CL diagnostic facilities are available.

Cutaneous leishmaniasis in Syria is diagnosed clinically and confirmed by the detection of amastigotes in a Giemsa-stained smear from lesion scraping examined under immersion. The patients who have lesions from which the parasites were detected by this method are termed *smear-positive*, and those in whose smears no amastigotes could be seen, *smear-negative*. For the purpose of this study, we also cultured biological material from the lesions. The patients from which promastigotes were detected in the culture medium will be termed *culture-positive* and those in whose cultures promastigotes could not be detected, *culture-negative*.

Our attempt to examine the demographic and clinical characteristics of leishmaniasis patients in Aleppo based on routinely collected data, as well as the relationship between them and the treatment course, is described in Chapter 2, where the weaknesses of each dataset is discussed. Thus, we aimed at examining the same relationships in a sample of patients which is smaller but followed up more closely.

The study proposal and data collection instruments were reviewed and approved by the Ethical Committees at LSHTM and Syrian Ministry of Health.

3.2. Materials and methods

Patient recruitment, examination and follow up was performed by one investigator. This imposed certain limitations on the work schedule. Cutaneous leishmaniasis is treated in Aleppo by intralesional injections of Glucantime® (meglumine antimoniate) once a week, so that patients who receive an injection on a certain weekday would normally receive their next injections on the same weekday.

Given this, during the work-week up to five health centres could be covered but it was decided to leave one day free for the laboratory work necessary for culturing the clinical isolates (see Chapter 4). For this reason, four health centres needed to be selected as study sites.

3.2.1. Sample size

Because no information was available on variation in time to cure, variation in drug sensitivity of *Leishmania tropica* or the association between treatment duration and parasite sensitivity, rather arbitrary assumptions had to be made. Since neither clinical nor drug sensitivity characteristics were known, the calculations were made to detect possible relationships between *Leishmania* drug sensitivity and clinical outcome. For the purpose of simplification, the sensitivity was categorised into tolerance and intolerance, the division between the two to be made either by median sensitivity as determined by the fifty-

percent effective concentration (EC_{50}) or, in case of bimodal distribution, according to the range of numbers in each group; and the clinical response into cure or non-cure at a set time point close to median treatment duration. Cure is defined in the section 3.2.5. *Baseline and follow-up data* below. The median treatment duration was expected to be roughly equal to one treatment course of six or eight doses. This duration was based on personal communications with Dr. Lama Jalouk (Syria) and Dr. Soner Uzun (Turkey).

Assuming that proportion of laboratory parasite tolerance in treatment failures equals 40% and in treatment successes 5%, EpiInfo (version 6, CDC, Atlanta, Georgia, USA) sample size calculator suggested two equal groups of 27 patients each. Assuming baseline culture positivity rate to be around 25% [Alrajhi *et al.*, 2002], about two hundred patients should be recruited. However, our baseline culture success rate approached 70% which allowed us to decrease the minimum sample size.

3.2.2. Selection of PHC centres for the study

To cover different study areas, two centres from the countryside and two from the city were to be selected. These centres were to be located in non-adjacent areas.

The number of patients reported by the health centres of Aleppo were examined (Appendix 2). Since it could be expected that an unknown proportion of patients might refuse to participate in the study, and of those who agree to participate an unknown proportion would drop out, the centres for the current study were selected that report large number of leishmaniasis patients.

However, some PHC centres would diagnose CL in patients from outside their catchment area, and then refer these patients to a PHC centre that treats CL near the patients' place of residence. These patients would be reported by the PHC centre that performed the diagnostic smear. For this reason, the centres that only have treatment, but not diagnostic, facilities, report no patients.

The centres that reported the highest numbers of patients were contacted and visited to obtain a clearer idea of the actual number of patients treated there, and the centres that mainly diagnosed and treated patients from their own catchment area, i.e. where the patients were more likely to continue treatment in the same PHC centre, were preferred. In the city, one of the candidate centres was closed for refurbishment, and another centre served a population which included a considerable proportion of immigrants from nearby villages who were likely to leave for their villages for several weeks during harvest seasons. In the countryside, Hritan centre, in fact, diagnosed cases from several nearby villages and referred them to local health centres for treatment, and these cases constituted about half of the reported patients.

Finally, the two centres selected in the city were Yousef al-Azmeh (in the north-east) and the Specialised Centre for Skin Diseases in Hamadaniyyeh (south-west), and in the countryside, PHC centres of Atareb (a town about 30 km to the WSW of Aleppo with population about 20,000) and Kafr Hamra (a village 7.5 km NW of Aleppo with a population about 9,000). The latter location was considered an emerging focus of CL, and treatment, but not diagnostic, facilities were introduced into its PHC centre in that year — a member of staff was trained to administer intralesional treatment for patients diagnosed elsewhere but no laboratory was equipped and no one of staff was trained to prepare and read smears. In the latter centre we introduced diagnostic services: once a week, during our visit, smears were prepared from the lesions of all the patients that sought diagnosis, and these smears were then taken to LCC where they were examined microscopically, and the results were reported to Kafr Hamra PHC so the patients might start treatment without having to leave their village for diagnosis. The population was informed about the availability of diagnosis on a certain weekday by announcements made from minarets.

3.2.3. Patient eligibility

The patients were eligible for the study if they agreed to participate, had not more than five lesions, had not received antimonial treatment for this instance of disease (i.e. patients with history of previous cured CL, whether treated or not, would be included if they were not treated for their current CL) and health centre staff deemed them eligible for intralesional treatment in this health centre. In addition, it was required that parasites be demonstrated in lesion smears and/or by culture, so the patients with negative smear results were considered conditionally eligible until culture results became available. If the culture became positive, the patient was contacted and invited to start treatment. If the staff made a decision to start treatment of a smear-negative patient on clinical grounds, the patient would be followed up. If during this provisional follow-up the patient became ineligible, they were excluded. For example, one patient who had five lesions and was negative by smear was contacted after her culture became positive, and when she came she presented four new lesions for a total of nine, and was excluded from the study.

3.2.4. Recruitment and consent

The purpose of the study and the expected procedures were explained to every patient or, in case of children, to their parent or accompanying adult, and any additional questions answered. It was also explained that the patient's decision would not affect in any way the quality of care, and that the patient is free to withdraw from the study at any time. The consent form also included points concerning the photographic images obtained in the course of treatment, and the patients had a chance to accept or refuse photography altogether or of certain lesions, and could accept or refuse possible specific uses of photographs, such as other research uses, teaching, publication in medical periodicals or publishing on the internet, provided the images would not allow the identification of the patient. Informed consent was obtained and the form

signed by literate adult patient or adult guardian (Appendices 3 and 4). If the patient or guardian were illiterate, the consent form was signed by two members of PHC centre staff who witnessed the consent.

The aim was to invite every new eligible patient to participate in the study. However, when the patients were too numerous during the peak season, those who came while another new patient was being interviewed, and the interview was unlikely to be finished soon, these candidates were not invited, so that minimum disruption was caused to patients and health centre staff. The maximum reasonable number of patients that could be recruited on a single day would not usually exceed six.

3.2.5. Baseline and follow-up data

After obtaining consent from the patient or accompanying adult, basic demographic, education and clinical presentation data were recorded in a specially designed questionnaire (Appendices 5 and 6). In addition, patient's telephone numbers were obtained. The adult patients or the adult accompanying minors were also questioned about their knowledge, attitudes and practices with regard to the disease (presented in Chapter 5).

Patients who missed an appointment or did not turn up until about noon were contacted by telephone. If they could not make today's appointment, they were asked to come in the next week.

The patient's lesion was measured at baseline, then before every injection during the course of treatment. Measurements were performed with a pair of dividers and a ruler. Two measurements were made for each lesion: the length (the longest diameter) and the width (the longest perpendicular to the length) of the outer palpable border of the lesion. If there was any redness surrounding the palpable lesion, this was considered beyond the lesion borders. The lesion was photographed with a digital camera (an eight-megapixel Sony DSC W-100), with a ruler with patient ID and date of visit visible in the frame.

For every injection, an attempt was made to assess the quality of drug administration.

Several authors agree that the drug should be injected (1) into upper and mid-dermis (2) from different sides of the lesion (3) using a fine-bore (insulin) needle; (4) new needle must be used for each injection; (5) complete blanching of the lesion must be achieved; (6) necrotised tissues should not be injected [Dowlati, 1996; Alkhawajah *et al.*, 1997; Gurei *et al.*, 2000; Ministry of Health (Syria), 2003; Blum *et al.*, 2004].

In practice, the above-mentioned points are emphasised in Aleppo, except using a new needle for each injection: when the patient needs more than one injection, they are all performed with the same needle, unless the syringe needs to be refilled: a used needle is not introduced into the drug vial or ampoule, which serves more than one patient.

Each of the five points was assessed, whenever possible, on a binary yes/no (1/0) scale, and a binary five-digit number was recorded in a special field for each treatment session.

For non-ulcerated lesions, the cure was defined as disappearance of the infiltrate, so that the lesion cannot be detected by palpation. For ulcers, the additional condition was complete re-epithelialisation of the ulcer surface. In all cases, the lesion must be judged as cured by the health centre staff who are actually responsible for patient management. Date of cure was the first date on which the patient was seen as cured. This might happen either during a treatment course or after a scheduled or non-scheduled treatment break.

3.2.6. Data entry

The data were entered into EpiData version 3.1 (The EpiData Association, Odense, Denmark) by two persons. The data from the questionnaires were dictated aloud by one person (Dr. J. Abazid, a dermatologist), and repeated aloud while being entered by the other (myself). The first person checked the

data while they were entered, then the next piece of data was dictated. EpiData files were exported to Microsoft Excel workbook format, readable by SPSS.

3.3. Results

3.3.1. Sample description

One-hundred thirty-two patients were enrolled in the study. Of these, twenty-five patients had lesions from which no parasites had been seen on smear or grown on culture (defined as *negative* both by smear and culture), and were excluded, leaving 107 patients in the sample. Of these, 81 patients had lesions from which parasites had been seen on smear and grown on culture (defined as *positive* by smear and culture), six positive by smear only and twenty by culture only. For patient inclusion purposes any culture with detectable promastigotes was counted, including those contaminated with bacteria, yeasts or moulds, that were later destroyed and discarded.

Demographic and clinical presentation characteristics of the patients included in and those excluded from the study are presented in Table 3.1. Included patients had significantly fewer lesions (mean, 2.13 *vs.* 2.68, median 2 *vs.* 3, p=0.031, Mann-Whitney U test), were more likely to be males (56.1% *vs.* 28%, p=0.011, Pearson's χ^2), more likely to have lesions on the face (53.3% *vs.* 20%, p=0.003, Pearson's χ^2) and less likely to have lesions on the lower extremities (16.8% *vs.* 30.6%, p=0.032, Pearson's χ^2). The differences in age, reported lesion duration at presentation, lesion locations on the upper extremities, trunk, ears or neck, lesion type (papule *vs.* ulcer) and the location of the primary health centre (urban *vs.* rural) were not statistically significant. Forty-five patients (34.1%) presented with one lesion.

The analysis that follows will be restricted to the patients that met our inclusion criteria, unless stated otherwise.

3.3.2. Demography

Patients' ages ranged between less than a year to seventy years. Due to relatively small number of patients, year-by-year description of age distribution was not feasible because most ages occurred only once or twice, and the maximum frequency was eight (for three-, nine- and ten-year-olds). The patients were divided into two groups: 0–14 and 15+ year old.

The age was significantly higher in the urban health centre patients compared to rural, but was not different between sexes (Table 3.2).

The proportion of males was higher in urban compared to rural centres but the difference was not statistically significant (62.0% *vs.* 50.9%, p=0.247, Pearson's χ^2).

The patients in Kafr Hamra centre were significantly younger than those in the other study locations (median age 7 *vs.* 14 years, p=0.0002, Mann-Whitney U test). The differences between the other centres were not significant (p=0.232, Kruskal-Wallis test).

3.3.3. Clinical presentation

3.3.3.1. Lesion location

More than half of the 107 eligible patients (57, or 53.3%) had lesions on the face, and fifty-five patients (51.4%) on the upper extremities. Lesions on the lower extremities were encountered in eighteen (16.8%) patients, and on the neck, ears and trunk in seven, five and three patients, respectively.

Lesion locations were not statistically different between sexes nor between health centres (urban *vs.* rural). For age groups, the younger age group was significantly more likely to have lesions on the face (67.6% *vs.* 28.2%, p<0.0001, Pearson's χ^2) and less likely to have lesions on the upper extremities (38.2% *vs.* 74.4%, p=0.0003, Pearson's χ^2). For the other locations, the differences were not statistically significant (Table 3.3).

3.3.3.2. Number of lesions

The mean number of lesions in our sample was 2.13, and the median, 2. The differences in numbers of lesions between sexes, younger and older patients or health centre locations were not statistically significant.

3.3.3.3. Lesion duration at presentation

The mean lesion duration in our sample was 2.74 months, and the median two months. The only statistically significant difference was between males and females, with the males reporting a shorter lesion duration than females (mean 2.51 *vs.* 3.04, median 2 *vs.* 3, p=0.011, Mann-Whitney U test). No significant differences by age, lesion location, number of lesions or health centre location were detected (Table 3.4).

3.3.3.4. Smear result

Since the positivity of smear or culture was one of our inclusion criteria, this analysis includes the recruited patients that were later excluded from the study due to negative smear and culture.

Mean and median values of continuous variables (age, number of lesions and reported lesion duration at presentation) in smear-positive and smear-negative patients were calculated and the significance of the differences examined using Mann-Whitney U test. Categorical variables (sex, health centre location and lesion location on the face *vs.* no lesions on the face) were cross-tabulated and the significance of the differences examined using Pearson's χ^2. No differences were statistically significant.

When the health centres were analysed separately, the smears were positive in 22/24 patients (91.7%) in Atareb, 13/16 patients (81.2%) at the Specialised Centre for Skin Diseases, 27/50 patients (54%) in Kafr Hamra and 26/42 patients (61.9%) in Yousef al-Azmeh, and these differences were statistically significant (p=0.006, Pearson's χ^2). The patients diagnosed at Kafr Hamra centre were significantly less likely to have a positive smear result compared to other

centres, although their smears were read by dedicated laboratory staff at the Leishmaniasis Control Center (p=0.016, Pearson's χ^2).

Lesion duration at presentation was shown in Chapter 2 to affect smear result, with highest positivity rates in lesion duration of two months. So lesion durations were categorised into three categories, of durations of one month or shorter, two months and three months or longer, and analysed by binary logistic regression with the lesions of one month or shorter selected as the reference category. The lesions of two months' duration were significantly more likely to result in positive smears (odds ratio 2.91, 95% confidence interval 1.04–8.15, p=0.041) compared to those of shorter durations, and lesions of three months or longer did not differ significantly from the reference category (odds ratio 1.11, 95% confidence interval 0.48–2.57, p=0.808).

3.3.3.5. Culture result

This section includes all the patients who were recruited, both those who were included in the final analysis and those who were excluded due to negative smear and culture results.

The analysis was conducted as for the smear result above. The results are presented in Table 3.5. The only significant association was lesion location on the face: the culture was positive in 87.1% of patients who had any lesions on the face *vs.* 65.7% of patients who had no lesions on the face (p=0.004, Pearson's χ^2).

Again, the Kafr Hamra health centre provided significantly less positive cultures, 66% *vs.* 81.7% in other centres (p=0.041, Pearson's χ^2). The differences between the other health centres were not statistically significant (p=0.184, Pearson's χ^2).

Analysing the relationship between lesion duration at presentation and culture result by logistic regression did not show statistically significant differences. Compared to the reference category with lesion durations of one

month or less, the patients with lesions of two month duration had odds ratio of positive result of 1.08 (95% CI 0.402–2.89, p=0.879).

The relationship between laboratory results and lesion type (papule *vs.* ulcer) could not be analysed because biological material was obtained from ulcers in only five cases, and of these two were positive by smear and three by culture, and only one patient was positive by both methods.

3.3.3.6. History of previous cutaneous leishmaniasis

Nine patients reported previous leishmaniasis which was treated in eight of them. The only untreated patient was a seventy-year-old lady who had leishmaniasis in childhood that cured spontaneously. Previous leishmaniasis occurred in the treated patients one to five years before the index lesions, and in one of them the new lesion appeared one month after she finished the treatment for the previous instance.

The patients who had previous leishmaniasis did not differ significantly from those who presented with the first instance of the disease with regard to age, lesion duration or treatment but the number of lesions was significantly higher in patients who reported no previous leishmaniasis (Table 3.6).

3.3.4. Clinical course

3.3.4.1. Description of the sample

Treatment duration and time to cure were calculated in the same way as in Chapter 2: treatment duration was obtained by subtracting the date of first treatment session from the date of the last treatment session. This gave the number of days of treatment. To convert to weeks, three was added to the result, then it was divided by seven and the integer was taken as number of weeks. Time to cure was calculated in a similar way, with the difference that the date of first treatment session was subtracted from the date when the cure was

diagnosed. This variable was available only for the patients who finished their follow-up until they were told by the health centre staff they were cured.

One hundred and seven patients met inclusion criteria. For these patients, the number of treatment sessions, treatment duration and time to cure is analysed.

Of these patients, nine (8.4%) did not start treatment (zero visits), and were excluded from further statistical analysis, so treatment duration and number of visits data were available for 98 patients.

Data on date of cure (and, hence, on time-to-cure) were available for fifty (46.7%) patients.

3.3.4.2. Description of clinical variables

For all patients who started treatment mean, median and mode for the number of treatment sessions, treatment duration and time to cure were calculated. In addition, for patients whose time to cure was known, the period between the last injection and the diagnosis of cure was calculated. This period will be referred to as *convalescence*. The latter three variables are expressed in weeks.

The number of treatment sessions and treatment duration were available for 98 patients, and time to cure and convalescence for fifty patients. The results are presented in Table 3.7.

The patients whose date of cure was known were compared to those who were lost to follow-up, and the results are presented in Table 3.8. The former are termed *complete follow-up* and the latter, *incomplete follow-up*. The patients with incomplete follow-up were significantly older (median, 15 *vs.* 9 years), had fewer lesions, attended significantly less treatment sessions and had significantly shorter duration of treatment. The differences in lesion duration at presentation, sex composition and lesion location (any lesions on the face *vs.* no lesions on the face) were not statistically significant.

3.3.4.3. Differences by age and sex

Mean and median values for the number of treatment sessions, treatment duration and time to cure were calculated and the medians compared between age groups (0–14 years and 15+ years) and sexes by Mann-Whitney U test. The results are presented in Table 3.9. Younger patients had more treatment sessions, longer treatment duration and time to cure and shorter convalescence compared to the older patients, and the differences were statistically significant (p=0.0001, 0.01, 0.037 and 0.027, respectively). Females had more treatment sessions, longer treatment duration and time to cure and shorter convalescence compared to males, but the differences were not statistically significant.

3.3.4.4. Differences by lesion location

This was analysed for all patients where those who had any lesions on the face were compared with those who had no lesions on the face, then for patients with one lesion, where location on the face was compared with other locations. The results are presented in Table 3.10.

In both groups the patients with lesions on the face received significantly more injections and their treatment duration was significantly longer. Time to cure was not significantly different. The convalescence period was shorter in those who had lesions on the face, but this was significant in all patients but not in patients with one lesion.

3.3.4.5. Differences by health centre

The differences between health centres in the number of treatment sessions and treatment duration were not statistically significant. However, the values for time to cure and convalescence were significantly different, with patients at Hamadaniyyeh (Specialised Centre for Skin Diseases) having the shortest median time to cure and Kafr Hamra the shortest convalescence period. Patients treated at Atareb had the longest values both for time to cure and convalescence. The results are presented in Table 3.11.

3.3.4.6. Differences by number of lesions

The number of lesions was categorised into "one or two lesions" and "three lesions or more" in the same way as in Chapter 2. This resulted in two groups of 69 and 29 patients for the first two outcomes and 39 and 11 patients for the last two outcomes, and the differences between them were not statistically significant: the median number of treatment sessions in patients with one or two lesions was nine *vs.* six in those with three lesions or more (p=0.173, Mann-Whitney U test), treatment duration 12 *vs.* 10 weeks (p=0.185), time to cure, 22 *vs.* 23 weeks (p=0.707) and convalescence, 4 *vs.* 5 weeks (p=0.101).

3.3.4.7. Differences by lesion duration

Lesion duration was grouped into three categories as in Chapter 2, "up to one month", "two months" and "three months or longer", then to two categories, "two months or shorter" and "three months or longer". Both groupings did not reveal any statistically significant effects on the outcome variables tested (Table 3.12)

3.3.4.8. Multivariate analysis

The variables which showed p values below 0.1 in the analyses above were entered into a multivariate logistic regression model. Outcomes were grouped into binary categories divided by the median value. For every explanatory variable univariate regression was compared to the multivariate model. The results are reported by outcome.

3.3.4.8.1. Number of treatment sessions (visits)

The results for the number of treatment sessions are presented in Table 3.13. The explanatory variables entered in the model were age group (0–14 and 15+ years), lesion location (any lesions on the face *vs.* no lesions on the face) and follow-up status (patients who were followed up until cure *vs.* those who were lost to follow up before cure was formally diagnosed). The reference groups

were the younger age group, patients who had no lesions on the face and patients with incomplete follow-up. In addition, health centres were entered in the model, with Yousef Azmeh centre selected as reference group.

Compared to univariate analysis, the odds ratios were considerably deflated (became closer to 1) and remained statistically significant for lesion location on the face and follow-up status, but not for age and health centres. This shows that lesion location and follow-up status do confound each other, but nevertheless each of them significantly affects the number of treatment sessions, and that the differences attributed to age in univariate analysis become not significant when possible confounders are taken into account. In the univariate model two centres significantly differed from the reference centre, but in the multivariate model the differences were no longer statistically significant, which suggests that the apparent differences in the number of treatment sessions between them could be explained by variations in sample characteristics.

3.3.4.8.2. Treatment duration

The results for treatment duration are presented in Table 3.14. The explanatory variables and the reference groups were the same as for the number of treatment sessions. In univariate analysis only the Hamadaniyyeh health centre was significantly different from the reference centre. In multivariate analysis the odds ratios for age, lesion location and follow-up status were considerably deflated, and those for the health centres considerably inflated, but only the differences by age group and lesion location remained statistically significant. This suggests these two variables are associated, and that they may explain the differences in follow-up status and between health centres.

3.3.4.8.3. Time to cure

Only two variables were associated with statistically significant differences in time to cure divided into two categories by the median value in univariate analysis ($p<0.1$): age and lesion duration at presentation. The results are presented in Table 3.15. Compared to univariate analysis, the odds ratio was marginally inflated for age group when corrected for lesion duration. The odds ratio for lesion duration was considerably inflated for lesion duration and became statistically significant (p value was 0.08 in univariate analysis and decreased to 0.045) indicating that, although the two variables confound each other, yet both are probably significant predictors of time to cure.

3.4. Discussion

The age distribution of our sample was closer to Treatment and Patient Cards datasets than to the leishmaniasis population of 2005 (the Main dataset in Chapter 2). However, unlike the three datasets examined in Chapter 2, in our sample males predominated. The mean number of lesions in our sample was 2.13 *vs.* 2.17 in the leishmaniasis population. The smear result was positive in 81.3% of cases *vs.* 78% in leishmaniasis population. Thus, our sample is fairly representative of the population, except for the sex distribution.

The patients included in the study differed from the excluded ones in their number of lesions (less in our sample), sex (more males in the sample) and lesion location (more lesions on the face and less on the lower extremities).

Lesion locations were not significantly different between urban and rural health centres or by sex, but younger patients were more likely to have lesions on the face and less likely to have lesions on the upper extremities. The same trend was noted in the leishmaniasis population for the age groups, and the differences between sexes, while statistically significant due to larger sample size, were not strikingly different.

While the differences in the number of lesions by age and sex were not significant in the sample, the same trend as in the population was observed with fewer lesions in males and the younger age group.

Lesion duration at presentation did not differ significantly between age groups, and the same trend as in the population was observed, with younger patients tending to present earlier. However, in our sample the females had significantly longer lesion durations in contrast with the population. This variable can be compared with the data from Turkey [S. Uzun, personal communication, 2005], where the median reported lesion duration at presentation was six months, which may reflect better awareness of the people in Aleppo of the disease, probably due to its high incidence, long history and health education efforts.

No significant relationships between the smear result and other possible explanatory variables were detected in our patients.

The mode for the number of treatment sessions was six, and for treatment duration five weeks. This corresponds to one standard treatment course and means that most patients stop treatment after one course of injections, either because they are cured or because they feel they need no more treatment. The mode value for time to cure may also be consistent with this, as after six injections the patients are asked to return after one month, or five weeks, for examination. However, the mode for convalescence was one week, which suggests that most frequently the cure is diagnosed in the middle of the course when the patient comes for their next injection and are told they are cured.

The patients who completed follow up were significantly younger than those who did not. This may be due to the fact that children may have no choice over whether to stop the treatment because they are brought to the health centre by adults, whereas the adult patients are more likely to decide to decline further treatment. This is further illustrated by less treatment sessions and shorter treatment duration in older patients. However, for the patients with

complete follow up, younger patients had significantly longer time to cure despite shorter convalescence. This suggests children tend to respond to intralesional antimonials less well.

The patients who completed follow up had significantly fewer lesions. This may be due to the fact that more lesions mean more injections in every session and, consequently, more pain. And, as expected, the patients with incomplete follow up had significantly less treatment sessions and shorter treatment duration.

Lesion location on the face was associated with significantly more injections and longer treatment duration. However, the differences in time to cure among patients who completed follow up were not statistically significant. This favours the hypothesis that lesion location on the face is a cause of more concern and an incentive to complete treatment and make sure the lesion is cured but the possibility that the lesions on the face need more treatment sessions or that health centre staff tend to treat them for longer periods cannot be excluded.

Multivariate analysis confirmed that age is a significant independent predictor of our outcome variables for all patients. Lesion location on the face was a significant independent predictor of number of treatment sessions and treatment duration but not time to cure. Follow up status was a significant independent predictor of number of treatment sessions only, and longer lesion duration at presentation was independently associated with shorter time to cure.

Our method of measuring the length and width of the lesions is not convenient for assessing the treatment course. The lesions typically enlarged upon treatment, then their dimensions remained relatively stable, but the lesion itself gradually became more flat (a dimension not measured in our study) until the infiltrate became no longer detectable by palpation.

Due to its relatively benign course, the Old World cutaneous leishmaniasis is a largely neglected disease. In most studies the causative agent is either not reported or is assumed based on previous results of epidemiological investigations. In addition, due to lack of consensus, the end-point of the studies vary considerably, making comparisons even more difficult [González *et al.*, 2008]. Very few studies were available for comparison, which differed in their aims and design. We review here the ones that were concerned with localised cutaneous *L. tropica* infection, where the causative organism was either typed or where it was known to be the only causative organism in the study area (namely, the Mediterranean and South-Eastern Anatolian Regions of Turkey, which are adjacent to Aleppo Governorate, and Kabul, Afghanistan) and which studied intralesional antimonial compounds either exclusively or as one of the study arms. In the current study, a selection of isolates obtained in Aleppo during fieldwork was typed, and all belonged to *L. tropica*. More details are reported in Chapter 4.

One study in Aleppo [Harms *et al.*, 1991], one in Afghanistan [Reithinger, Mohsen *et al.*, 2005], two from Iran [Hadighi *et al.*, 2006; Sadeghian *et al.*, 2007] and two from Turkey [Gürel *et al.*, 2000; Uzun *et al.*, 2004] are reviewed.

The demographic and presentation variables are compared to the group of patients that met our inclusion criteria.

Our patient sample has the lowest mean age among those reported: 16.3 years *vs.* 21 by Harms, 22.4 by Sadeghian and 22.4 by Uzun. Reithinger reported the median age (13 years), which is also greater than in our sample (11 years). This may indicate longer endemicity of the disease in Aleppo if the development of immunity is assumed.

The number of lesions in our sample was greater than that reported by other authors: in our sample the mean number of lesions was 2.1, while Sadeghian reported 1.5, Uzun, 1.4 and Gürel, 1.4. In our sample 38% of patients

presented with one lesion, while the proportion was 63% in the sample of Hadighi and 81% and 80% in the studies of Uzun and Gürel, respectively.

The median lesion duration at presentation was reported by Reithinger (2005) and Uzun (personal communication), and it was six months in both studies, compared to two months in our sample. Sadeghian reported a mean lesion duration of four weeks. In our sample the mean lesion duration was 2.7 months.

Eighty-eight percent of our patients presented with non-ulcerative lesions, and 94% of all lesions were non-ulcerative. Both Hadighi and Uzun reported 71% of non-ulcerative lesions. In the case of Uzun, this might be due to longer average lesion duration at presentation, a variable not reported by Hadighi. Sadeghian reported no ulcers among his patients which might be due to unusually short lesion durations in his sample.

Some studies reported baseline lesion size as maximum lesion diameter. In our sample the mean and median lesion diameter was 9.1 mm and 7 mm, respectively. Reithinger reported median value of 12 mm, Sadeghian, a mean of 14.7 mm, Uzun, a mean of 13.6 mm and Harms, a median of 15 mm, and it was usually stated that the measurements were made to the infiltrate, not the surrounding redness, so the data are comparable. Our sample showed the smallest baseline lesion size. This may be related to earlier presentation of our patients, although the patients of Sadeghian presented with considerably shorter lesion durations yet had larger lesions.

Most studies reported proportion of patients that were cured at a fixed point of time rather than time to cure. Similar proportions could be calculated for our patients in most cases. For this purpose, the patients who completed follow-up were selected. Reithinger reported 50% cure at 75 days and 75% cure at 100 days. Only 22% and 40% of patients were cured at these time points, respectively, in our sample. Hadighi and Sadeghian reported percent of cure at six months, 90% by the former and 57% by the latter. In our sample 74% of

patients were cured at week 26. Interestingly, Sadeghian reported two measures of cure: proportion of patients cured and proportion of lesions cured, and both were in perfect agreement. Harms reported 76% cure rate after ten weeks, compared to 18% in our sample. Finally, Gürel reported 85% cure rate both after thirty and ninety days. In our sample only one patient (2%) was cured at five weeks and 32% at thirteen weeks, the closest point to ninety days. Uzun reported mean and median number of injections in their study, which were equal to 8.5 and 7, respectively. In our study the corresponding values among patients with complete follow up was 9.5 and 8, respectively. Thus, our numbers were consistently higher than those reported by other authors. However, these data cannot be compared directly due to important differences in treatment regimens. Most studies used regimens of four or five doses given in weekly intervals (in case of Reithinger, every five or seven days), Uzun in Turkey used continuous course of weekly injections until cure for a maximum of twenty weeks. In Aleppo, injections are given weekly in courses of six, and after each course there is a one-month (in practice, five-week) break. It is not infrequent that in the end of the course the lesion is not fully cured. In such cases the patients may be asked to come back after another month for evaluation and possible continuation of treatment or formal discharge. For this reason our time to cure variable may be longer than actual time to cure, and this is illustrated by the delay between the last injection and diagnosis of cure, that we termed 'convalescence', which was about a month on average (with mean of 5 weeks and median of 4 weeks). However, the mode value was one week, which means many patients were told they were cured when they turned up for their next injection during a treatment course.

In summary, the characteristics of cutaneous leishmaniasis in Aleppo appear different from other settings. Possible explanations for these differences may lie in the causative organism, host population characteristics and in the history of the disease, as Aleppo is a well-known long-standing focus of

anthroponotic cutaneous leishmaniasis, and there is a relatively strong, comprehensive and well-managed programme of leishmaniasis control which is based primarily on the treatment of passively-detected cases.

A considerable proportion of patients do not complete the treatment until they are formally told they are cured. Pentavalent antimonials are capable of selecting resistant parasites in experimental models [Grögl *et al.*, 1989; Lucumi *et al.*, 1998], The parasites do not disappear from the lesions promptly but can still be detected in some patients undergoing treatment. It can be speculated that in the lesions of patients who prematurely stop the treatment the parasites will multiply again, and these patients will serve as potential source of infection by less antimony-sensitive parasites. There are reports of isolation of less sensitive parasites from patients with treatment failure or subcurative treatment with antimonials [Grögl *et al.*, 1992; Rojas *et al.*, 2006].

Since the humans are believed to be the only source of anthroponotic cutaneous leishmaniasis infection, treatment-selected antimony-resistant parasites may be transmitted to other people and cause infections with insensitive parasites (often termed by clinicians 'primary resistance').

Thus, it is worthwhile to explore the sensitivity of *Leishmania* to antimonial preparations in Aleppo and whether any relationships can be found between parasite sensitivity and clinical outcome of the disease. We attempted to isolate the parasite from leishmaniasis patients in Aleppo before and during the course of treatment. This is described in the next chapter.

3.5. Tables

Table 3.1. Demographic and clinical presentation characteristics of the patients included in (n=107) and excluded from (n=25) the study.

Variable	Included		Excluded		p
	mean	*median*	*mean*	*median*	
Age, years	16.28	11	18.76	12	0.379*
Number of lesions	2.13	2	2.68	3	0.031*
Lesion duration, months	2.74	2	2.42	2	0.471*
Sex, % males	56.1		28.0		0.011†
Lesions on face, % of patients	53.3		20.0		0.003†
Lesions on hand, % of patients	51.4		60.0		0.438†
Lesions on leg, % of patients	16.8		30.6		0.032†
Lesions on trunk, % of patients	2.8		4.0		0.753†
Lesions on ears, % of patients	4.7		4.0		0.884†
Lesions on neck, % of patients	6.5		0.0		0.189†
Centre location, % rural	53.7		66.7		0.247†
Ulcers, number/total (%)	13/107 (12.1)		2/25 (8)		0.556†

* Mann-Whitney U test; † Pearson's χ^2. For lesion locations the sums add to more than 100% because some patients had lesions in more than one location.

Table 3.2. Mean and median age (years) comparisons: males *vs.* females and rural *vs.* urban health centres.

	n	mean	median	p*
males	60	14.32	10	0.290
females	47	18.79	12	
urban	50	18.56	13.5	0.047
rural	57	14.28	9.0	

* Mann-Whitney U test; n, number of patients in each group.

Table 3.3. Lesion location by sex, age group and health centre location. The values in the cells are percentages of patients in each category having lesions in the corresponding location.

Location	males	females	p*	0–14	15+	p*	urban	rural	p*
Face	53.3	53.2	0.988	67.6	28.2	<0.0001	52.0	54.4	0.805
Hand	46.7	57.4	0.268	38.2	74.4	0.0003	56.0	47.4	0.373
Leg	16.7	17.0	0.961	13.2	23.1	0.190	24.0	10.5	0.063
Trunk	1.7	4.3	0.421	2.9	2.6	0.909	2.0	3.5	0.637
Ears	5.0	4.3	0.856	7.4	0.0	0.083	6.0	3.5	0.542
Neck	5.0	8.5	0.466	7.4	5.1	0.654	8.0	5.3	0.568

* Pearson's χ^2.

Table 3.4. Reported lesion duration at presentation by sex, age, lesion location, number of lesions and health centre location.

	n	mean	median	p*
males	59	2.51	2	0.011
females	46	3.04	3	
0–14	68	2.53	2	0.234
15+	37	3.14	2	
lesions on face	56	2.50	2	0.199
no lesions on face	49	3.02	2	
1 or 2 lesions	72	2.75	2	0.910
3 lesions or more	33	2.73	2	
urban	49	3.02	2	0.407
rural	56	2.50	2	

* Mann-Whitney U test; n, number of patients in each group.

Table 3.5. Relationship between culture result and age, number of lesions, lesion duration at presentation, sex, lesion location and health centre location. The differences between health centres are reported in the text.

Culture	Positive		Negative		p
	mean	*median*	*mean*	*median*	
Age, years	16.04	10	18.97	14	0.228*
Number of lesions	2.15	2	2.50	2	0.199*
Lesion duration, months	2.81	2	2.29	2	0.217*
Sex, % males	55.0		37.5		0.085†
Lesions on face, % of patients	54.0		25.0		0.004†
Centre location, % rural	54.0		62.5		0.399†

* Mann-Whitney U test; † Pearson's χ^2.

Table 3.6. Comparison between the patients with (n=9) and without (n=98) history of previous leishmaniasis.

Previous leishmaniasis	No		Yes		
	mean	*median*	*mean*	*median*	p*
Age, years	15.89	10	20.56	12	0.283
Number of lesions	2.19	2	1.44	1	0.027
Lesion duration, months	2.77	2	2.38	1.5	0.506
Treatment duration, weeks	14.18	12	13.12	9.5	0.886

* Mann-Whitney U test.

Table 3.7. Mean, median and mode values for treatment sessions, treatment duration, time to cure and convalescence. The first two variables were available for 98 patients, the latter two for 50 patients. The latter three variables are expressed in weeks.

Variable	mean	median	mode
Treatment sessions	9.5	8	6
Treatment duration	14.1	12	5
Time to cure	21	22.5	10
Convalescence	5	4	1

Table 3.8. Comparison of demographic and clinical characteristics of patients with complete and incomplete follow up.

	Follow up				
	incomplete		complete		
	mean	*median*	*mean*	*median*	p
Age (years)	21.4	15	12.0	9	0.029*
Number of lesions	2.4	2	1.9	2	0.044*
Lesion duration (months)	2.6	2	2.8	2	0.509*
Treatment sessions	7.9	6	10.9	10	0.001*
Treatment duration (weeks)	12.0	8	15.9	15	0.015*
Sex (% males)	60		54.7		0.598†
Lesion location (% face)	46.7		60.4		0.175†

* Mann-Whitney U test. † Pearson's χ^2.

Table 3.9. Mean and median values for treatment sessions, treatment duration, time to cure and convalescence by age group and sex.

	Age group				
	0–14		15+		
	mean	*median*	*mean*	*median*	p*
Treatment sessions	11.0	10	7.1	8	0.0001
Treatment duration	16.4	17	10.2	7	0.01
Time to cure	22.5	25	16.9	13	0.037
Convalescence	4.6	3	6.2	5	0.027
	Sex				
	M		F		
	mean	*median*	*mean*	*median*	p*
Treatment sessions	8.8	7	10.5	10	0.85
Treatment duration	13.4	10.5	15.1	12.5	0.390
Time to cure	19.8	18	22.5	25	0.195
Convalescence	5.3	4	4.7	4	0.493

* Mann-Whitney U test

Table 3.10. Mean and median values for treatment sessions, treatment duration, time to cure and convalescence by lesion location in all patients (any lesions on face *vs.* no lesions on face) and in patients with one lesion (n=37 for number of sessions and treatment duration, n=20 for time to cure and convalescence; face *vs.* other locations).

	All patients				
	any lesions on face		no lesions on face		
	mean	*median*	*mean*	*median*	p*
Treatment sessions	11.3	12	7.4	6	0.0001
Treatment duration	17.2	19	10.4	9	0.0002
Time to cure	21.2	23	20.8	22	0.726
Convalescence	3.9	3	6.8	5	0.019
	Patients with one lesion				
	on face		in other locations		
	mean	*median*	*mean*	*median*	p*
Treatment sessions	11.5	10	6.4	6	0.003†
Treatment duration	18.0	22	9.7	8.5	0.029†
Time to cure	21.9	25.5	18.3	17	0.473*
Convalescence	3.3	2.5	7.3	5.5	0.157†

* Mann-Whitney U test. † Exact significance is reported due to small numbers of patients.

Table 3.11. Mean and median values for treatment sessions, treatment duration, time to cure and convalescence by health centre.

	Health centre								
	Atareb		*Kafr Hamra*		*Hamadaniyyeh*		*Yousef Azmeh*		p*
	mean	*med*†	*mean*	*med*†	*mean*	*med*†	*mean*	*med*†	
Treatment sessions	9.2	7	10.5	10	6.7	6	10.3	10	0.052
Treatment duration	14.6	11	13.5	12	9.1	6	16.7	17.5	0.052
Time to cure	28.9	29	16.8	15	13.75	11.5	22.5	24.5	0.009
Convalescence	9.8	8	3.1	1.5	5.5	5	4.3	4	<0.001

* Kruskal-Wallis test. † median.

Table 3.12. Mean and median values for treatment sessions, treatment duration, time to cure and convalescence by lesion duration at presentation.

	Lesion duration (months)				
	1 or 2		3 +		
	mean	*median*	*mean*	*median*	p*
Treatment sessions	9.4	8	9.9	10	0.759
Treatment duration	14.4	12	14.0	12	0.795
Time to cure	23.8	25	18.7	18	0.064
Convalescence	6.0	5	4.1	4	0.298

	≤1		2		3+		
	mean	*median*	*mean*	*median*	*mean*	*median*	p†
Treatment sessions	9.1	8	9.7	8	9.9	10	0.875
Treatment duration	14.4	12	14.4	14	14.0	12	0.965
Time to cure	20.8	23	26.6	26	18.7	18	0.080
Convalescence	5.0	3.5	6.9	5	4.1	4	0.345

* Mann-Whitney U test. † Kruskal-Wallis test.

Table 3.13. Multivariate regression model for the number of treatment sessions split into binary variable by the median value (≤7 *vs.* 8+). Younger age group, no lesions on the face, patients with incomplete follow-up and Yousef Azmeh health centre were the reference categories.

	univariate		multivariate	
Variable	**OR**	**p**	**OR**	**p**
Age group (≤14 *vs.* 15+)	0.18 (0.07–0.43)	<0.001	0.44 (0.15–1.33)	0.146
Lesion location (face)	6.81 (2.80–16.57)	<0.001	5.10 (1.73–15.03)	0.003
Follow-up status	3.81 (1.64–8.83)	0.002	3.16 (1.12–8.89)	0.029
Health centre				
Atareb	0.71 (0.24–2.09)	0.538	1.51 (0.40–5.70)	0.542
Hamadaniyyeh	0.18 (0.04–0.76)	0.019	0.29 (0.06–1.41)	0.123
Kafr Hamra	3.42 (1.04–11.32)	0.044	3.05 (0.77–12.12)	0.114

Table 3.14. Multivariate regression model for treatment duration split into binary variable by the median value (≤11 *vs.* 12+ weeks). Younger age group, no lesions on the face, patients with incomplete follow-up and Yousef Azmeh health centre were the reference categories.

	univariate		multivariate	
Variable	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Age group (≤14 *vs.* 15+)	0.24 (0.01–0.57)	0.001	0.33 (0.12–0.95)	0.039
Lesion location (face)	4.69 (1.99–11.02)	<0.001	3.41 (1.28–9.06)	0.014
Follow-up status	2.29 (1.02–5.15)	0.046	1.79 (0.70–4.59)	0.226
Health centre				
Atareb	0.48 (0.16–1.44)	0.19	0.79 (0.22–2.78)	0.707
Hamadaniyyeh	0.18 (0.05–0.67)	0.011	0.25 (0.57–1.11)	0.068
Kafr Hamra	0.56 (0.20–1.61)	0.285	0.34 (0.10–1.12)	0.075

Table 3.15. Multivariate regression model for time to cure split into binary variable by the median value (≤22 *vs.* 23+ weeks). Younger age group and lesion duration of two months or less were the reference categories.

	univariate		multivariate	
Variable	**OR (95% CI)**	**p**	**OR (95% CI)**	**p**
Age group (≤14 *vs.* 15+)	0.21 (0.05–0.87)	0.032	0.18 (0.04–0.91)	0.038
Lesion duration (≤2 *vs.* 3+)	0.36 (0.11–1.16)	0.08	0.27 (0.07–0.97)	0.045

3.6. Figures

Figure 3.1. The road signs on the entrance of the rural localities.

Chapter 4. Parasite sensitivity to antimonial drugs

4.1. Introduction

4.1.1. Background

As mentioned in the *Conclusion* to Chapter 1, in mid-1990s it was noted that cutaneous leishmaniasis in Aleppo required more intralesional injections to achieve cure than in previous years [Douba *et al.*, 1997]. The authors hypothesised that the lack of response they observed might have been due to several factors: selection of tolerant strains of parasites, inadequacy of intralesional treatment alone, technical errors in administration, failure to follow treatment protocol by medically unqualified persons in the informal sector or lack of patient compliance. The authors argue these factors would lead to partial immunity and to an increase in chronic lesions and, as a consequence, to an increase of human reservoir, and might also serve as an explanation for the increase in reported incidence of the disease. The authors were not able to conduct assays of parasite drug sensitivity due to lack of facilities and recommended that such assays be conducted in the future.

In this text *sensitivity* refers to inhibition of *Leishmania* isolates by the drugs in *in vitro* tests.

Treatment outcome depends on several factors related to the pathogen, the host and the treatment.

- Treatment factors may be drug-related or physician-related.
- Pathogen factors include innate or acquired tolerance to the treatment and virulence of the infecting strain(s).
- Host factors include immune response which might be adequate, suppressed or inefficient, or pathologically uncontrolled [Murray *et al.*,

2006]; treatment compliance is another important factor [Douba *et al.*, 1997].

- Drug-related factors are related to drug characteristics such as pharmacokinetics and quality.
- Physician-dependent factors include dosage, frequency, mode of administration, including possible technical errors in administration.

Anthroponotic infections have a higher potential of developing tolerance to anti-infectious agents due to drug pressure during treatment compared to zoonoses [Bryceson, 2001], unless animals are treated with the same drugs as humans [Dujardin *et al.*, 2008]. This is because in zoonoses humans are "dead-end" hosts, and drugs do not affect parasite's reproductive success because the parasite is not returned to the transmission cycle [Woolhouse *et al.*, 2002]. Therefore, it is important to explore whether the treatment used in Aleppo, where the disease is believed to be anthroponotic [Ashford *et al.*, 1993], leads to selection of less sensitive parasites. Development of *Leishmania* drug tolerance during treatment has not been explored extensively. While development of secondary tolerance (the one that develops during treatment) was demonstrated in a Colombian study in four of twenty patients with leishmaniasis caused by the *Viannia* subspecies of *Leishmania* [Rojas *et al.*, 2006], in a larger study of 185 clinical isolates from Iran, mostly belonging to *L. tropica*, only primary tolerance (when the isolates are initially tolerant to the drug) was found [Hadighi *et al.*, 2007].

4.1.2. Drug sensitivity determination

Sensitivity of *Leishmania* to drugs can be determined using promastigotes [Azeredo-Coutinho *et al.*, 2007], intracellular amastigotes [Berman *et al.*, 1982; Neal and Croft, 1984], axenic amastigotes [El Fadili *et al.*, 2005] or animal models [Escobar *et al.*, 2001; Sacks and Noben-Trauth, 2002], and measurement of sensitivity may be by counting infected

macrophages and/or parasites, radiolabelling or measurement using luciferase [Berman and Gallalee, 1985; Hadighi *et al.*, 2006].

Promastigotes are far less sensitive to pentavalent antimony preparations than amastigotes [Azeredo-Coutinho *et al.*, 2007]. This may or may not apply to other drugs (*see* "Overview of the drugs" below). For this reason, and because the amastigotes in the vertebrates are located intracellularly, determining *in vitro* sensitivity requires using a dividing population of the parasite stage found in mammal host [Croft and Brun, 2003]. A few studies explored the correlation between the sensitivity of promastigotes and amastigotes on one hand, and treatment outcome, on the other. While a study of treatment failures in kala-azar in India [Lira *et al.*, 1999] found a strong correlation between clinical response and *in vitro* sensitivity of *L. donovani* to sodium stibogluconate in amastigote, but not promastigote, assays, another study from Brazil found a positive correlation between drug sensitivities of *L. braziliensis* isolates in both stages, which also correlated with the clinical outcome (although promastigotes required drug concentrations higher by about two orders of magnitude compared to amastigotes) [Azeredo-Coutinho *et al.*, 2007], which might indicate that in different species of *Leishmania* there may or may not be correlation between sensitivities of the two parasite stages. However, both studies were done on small samples of patients (26 in the former and 19 in the latter).

The protocols for determining *in vitro* amastigote sensitivity to Sb^V vary widely with regard to length of incubation and exposure, number of duplicates for each drug concentration, number of concentrations used and drug dilutions in the series (Table 4.1).

4.1.3. Typing of *Leishmania*

The methods of *Leishmania* classification and typing are reviewed in greater detail in Chapter 1. In this chapter, the specific methods used for typing of the study strains are presented later (*Leishmania* typing, p. 168).

Several methods are applied for molecular characterisation of *Leishmania*. PCR-RFLP of cp8 and gp63, sequencing of ITS1 and multilocus sequence typing of housekeeping genes are suitable for species identification, whereas multilocus microsatellite fragment analysis and kinetoplast DNA typing are suitable for distinguishing single strains, the former can also be used to infer population structure [Schönian *et al.*, 2008].

4.1.4. Overview of the drugs

This brief overview is concerned with the drugs used in the assays. For each drug the available data on indications, sensitivity of different species of *Leishmania* (promastigotes and amastigotes) and pharmacokinetics are reviewed. This section is mainly based on Dollery's and Martindale reference books [Dollery and Boobis, 1991; Sweetman, 2002], unless stated otherwise.

4.1.4.1. Pentavalent antimony

Sodium stibogluconate (SSG) is a gluconate of antimonic acid. It is a mixture of ionic antimony compounds with ill-defined composition that has Sb^V content not below 30% and not above 34%, usually with less than two atoms of sodium per atom of antimony. The commercial solution of SSG contains 100 mg Sb^V/ml, of meglumine antimoniate (MGA), 81 mg Sb^V/ml. The two drugs are considered equivalent if the dose is calculated based on antimony content.

SSG and MGA are primarily used for treatment of leishmaniases. They are administered intramuscularly or intravenously at a dose of 20 mg/kg/day with no upper limit for at least twenty days. This dosage is used in Syria to treat cutaneous leishmaniasis (CL) that cannot be treated with intralesional injections.

Intralesional treatment is used to treat CL in the Old World. Treatment protocols vary, examples are: three injections on alternate days every month, repeated as necessary in India [Sharma *et al.*, 2005]; three injections a week for a total of ten injections or weekly injections in continuous course until cure in Turkey [Ok *et al.*, 2002]; weekly injections given in up to three six-week or eight-

week courses with one-month intervals between the courses in Syria, and a fortnightly regimen was successfully tested in Pakistan [Mujtaba and Khalid, 1999]. A substantial proportion of CL treatment research is poorly designed [Khatami *et al.*, 2007; González *et al.*, 2008].

Sensitivities of different species to Sb^V vary widely. Old-World "cutaneous" species seem to be less sensitive in amastigote-macrophage tests (most values >20 μg Sb^V/ml) compared to New-World species and *L. donovani* (most values <10 μg Sb^V/ml) [Neal *et al.*, 1995]. In one study comparing the sensitivities of promastigotes and amastigotes of Latin American species to Sb^V the sensitivity of promastigotes was lower by approximately two orders of magnitude, and there was positive correlation between the two [Azeredo-Coutinho *et al.*, 2007].

Pentavalent antimony compounds have poor gastrointestinal absorption. After intravenous administration, the initial distribution phase is followed by quick biexponential renal elimination. Elimination half-life of the initial phase is about 1.7 hours and that of the terminal phase about 33 hours. Peak concentration occurred one hour after administration of 50–100 mg Sb^V was 10–15 μg Sb^V/ml. About 80% of antimony is recovered in the urine after 6 hours, and up to 94% in 24 hours.

In one study [Chulay *et al.*, 1988] that examined pharmacokinetics after intramuscular administration of 10 mg Sb^V/kg, patients were treated for thirty days with either SSG or MGA, and the pharmacokinetics of both drugs were remarkably similar. The peak concentration of about 10 μg Sb^V/ml was reported after two hours. Initial absorption phase was characterised by a half-life of 0.85 hours and initial-phase elimination half-life was two hours. The mean half-life of the slow elimination phase was 76 hours. The authors attributed the slow elimination phase to partial conversion of Sb^V to Sb^{III}. A slow increase of trough concentrations during the course of treatment was noted.

One study measured antimony pharmacokinetics in blood and normal and affected skin of leishmaniasis patients. After intramuscular injection of SSG

equivalent to 600 mg Sb^V, the maximum concentration in blood (8.81 μg Sb^V/ml) was achieved after about an hour and a half, and in skin lesions, 5.2 μg Sb^V/ml after 2.1 hours [al Jaser *et al.*, 1995]. This dose corresponds to the low-dosage treatment protocol of 10 mg Sb^V/kg body weight, which may be suitable for *L. major* because it is generally sensitive to antimony. However, the World Health Organization (WHO) recommended a dose of 20 mg Sb^V/kg body weight for parenteral treatment with no maximum dose. Earlier, WHO recommended that the dose should not exceed 850 mg Sb^V [Alvar *et al.*, 1997].

Pharmacokinetics in children (aged between three and six years) were compared to adults in one study in patients with cutaneous leishmaniasis [Cruz *et al.*, 2007]. The patients were given MGA at 20 mg Sb^V/kg intramuscularly for 20 days. In addition, one group of children received 30 mg Sb^V/kg on the last day. Drug exposure was assessed by the area under the 24-hour time-concentration curve ($AUC_{0\text{-}24h}$) in plasma. The values in children were lower by 42% due to significantly higher (by 75%) weight-adjusted clearance. After a dose of 30 mg Sb^V/kg in one group of children, drug exposure reached 86% (corresponding to 48% increase compared to the standard, 20 mg Sb^V/kg, dose), and peak serum concentration 113% of adult values.

No studies were identified that examine the kinetics of tissue concentration of drugs following intralesional administration. These would be more relevant to the Syrian setting, as intralesional treatment with Glucantime is used as first-line treatment in Syria.

4.1.4.2. Amphotericin B

Amphotericin B is an antifungal polyene antibiotic administered intravenously as an antifungal or antiprotozoan treatment or applied locally for superficial fungal infections in immunocompetent patients.

In one study [Escobar *et al.*, 2002], sensitivity to amphotericin B of promastigotes and amastigotes of reference strains of different species of *Leishmania* was determined using amastigote–macrophage assay. Promastigotes

were on average as sensitive to amphotericin B as amastigotes, and EC_{50} values for *L. tropica* reference strain intracellular amastigotes were 0.05 and 0.09 μM in two experiments. In the second experiment infection levels in control wells were rather low (46%). For the other species tested the EC_{50} values for intracellular amastigotes were in the range between 0.036 (*L. donovani*) and 0.14 μM (*L. mexicana*), and for promastigotes, between 0.003 (*L. donovani*) and 0.27 μM (*L. mexicana*).

Amphotericin B is available in several formulations. The colloidal ("conventional", deoxycholate) formulation achieves plasma concentrations between 0.5 and 4 μg/ml, and on maintenance doses of 400–600 μg/kg is usually around 0.5 μg/ml. Half-life is about 24 hours. The metabolic pathways are unknown. About 40% is recovered unchanged in the urine. The drug binds with plasma proteins (90–97%).

Conventional amphotericin B is associated with nephrotoxicity that occurs in almost all patients. Glomerular and tubular damage is observed. The nephrotoxicity becomes irreversible in 15% of patients who received a total dose of 30 mg/kg and in 80% after a total dose of 75 mg/kg [Davidson and Croft, 1993].

Non-conventional formulations (lipid-associated) provide higher plasma and several times lower renal concentrations, and have better safety profile than conventional formulations [Lachaud *et al.*, 2009] but their use is limited in most settings by their high cost [Ameen, 2007], which may be offset by shorter hospital stays in Israel [Solomon *et al.*, 2007] and Europe but no other endemic region [Murray *et al.*, 2006]. They have a major advantage in visceral leishmaniasis because the liposomes are cleared by phagocytising mononuclear cells, and the drug accumulates in reticuloendothelial system, thus targeting *L. donovani*. Total doses of up to 15 mg/kg are not usually associated with nephrotoxicity. The drug should not be exposed to temperatures below 0°C or

above 25°C, as this causes changes to the liposomes and may increase toxicity or decrease efficacy [Bern *et al.*, 2006].

In a short report [Bau *et al.*, 2003] it was suggested that heating conventional amphotericin B to 70°C for 20 minutes leads to superaggregation and several-fold decrease of *in vitro* and *in vivo* toxicity and an increase of antileishmanial activity in mice, and that clinical trials be conducted. However, no further development of this suggestion could be traced.

The bioavailability after oral administration is less than 5%, which permits topical use for the treatment or prevention of fungal infections of the oral cavity and gastrointestinal tract with negligible toxicity [Meis and Verweij, 2001].

A recent study of a novel formulation of amphotericin B for oral administration [Wasan *et al.*, 2009], conducted on mice infected with *L. donovani*, reported promising results: the formulation given twice daily for five days at 10 or 20 mg/kg led to near-complete (>99%) suppression of parasite load in the liver. No toxicity data were reported.

4.1.4.3. Miltefosine

Miltefosine belongs to the alkylphosphocholine group of phospholipid analogues [Croft *et al.*, 2003]. It was originally developed as an anti-cancer drug but showed marked toxicity below therapeutic doses. Six-percent miltefosine solution was evaluated as topical application for skin metastases of breast cancer with limited efficacy [Terwogt *et al.*, 1999; Leonard *et al.*, 2001] and tried in skin lymphoma and melanoma. A single-case report was published about successful treatment with the same solution of skin metastases of epidermoid carcinoma [Mahieu-Renard *et al.*, 2005].

Miltefosine is the only oral preparation with demonstrated efficacy in the treatment of visceral leishmaniasis and Latin American cutaneous leishmaniasis [Berman *et al.*, 2006]. A study on mice suggested topical application of the 6% solution might be a promising approach for cutaneous

leishmaniasis [Schmidt-Ott *et al.*, 1999] but a trial in humans had discouraging results [S. L. Croft, personal communication].

In the study mentioned above [Escobar *et al.*, 2002], sensitivity to miltefosine of promastigotes and amastigotes of different species of *Leishmania* was determined using the amastigote–macrophage assay. Promastigotes were more sensitive to miltefosine than amastigotes, and EC_{50} values for *L. tropica* reference strain amastigotes were 5.82 and 10.83 μM in two experiments.

Pharmacokinetics of miltefosine were not studied extensively. In a relatively large trial cutaneous leishmaniasis due to *L. major* acquired in northern Afghanistan in 31 Dutch military personnel was treated with 150 mg miltefosine daily for 28 days, and the median plasma concentration in the last week of treatment was ~31 mg/ml (~75 mM), with a terminal elimination half-life of 30.9 days [Dorlo *et al.*, 2008]. The drug could be detected in plasma samples taken five to six months after the end of treatment, and the authors suggested the presence of subtherapeutic concentrations may contribute to selection of resistant parasites and emphasised the importance of measures to prevent the risk of teratogenic effect. The patients in this study either did not benefit from Pentostam and cryotherapy or had extensive disease. The outcome of miltefosine treatment was not reported.

4.1.4.4. Paromomycin

Paromomycin (aminosidine) is an aminoglycoside antibiotic that shares many features with other aminoglycosides but is active against several protozoa (*Leishmania, Entamoeba histolytica, Cryptosporidium* spp.) and tapeworms.

Oral absorption of paromomycin is low. It is administered intramuscularly for systemic treatment of visceral leishmaniasis [Sundar *et al.*, 2007]. It was recommended for the treatment of cutaneous leishmaniasis in the form of intralesional injections or topical formulations in the USSR [Shuvalova, 2001], and is used in methylbenzethonium chloride ointment in Israel [El-On *et al.*, 1992; Davidson *et al.*, 2009].

Tests in amastigote–macrophage model showed marked differences between species, the most sensitive (EC_{50}<5 μM) being *L. major*, *L. tropica* and *L. panamensis*. The EC_{50} of *L. amazonensis* was beyond the highest testing concentration. *Leishmania donovani* strains were mostly sensitive with EC_{50} values between 6.1–43.8 μM, with the exception of one strain (DD8) with EC_{50}>150 μM.

Pharmacokinetics of paromomycin were studied by Kanyok *et al.* in 1997 in healthy American volunteers [Kanyok *et al.*, 1997]. Two doses, 12 or 15 mg/kg intramuscularly, were tested. The mean peak plasma concentration was 22.4 μg/ml (approximately 36 μM) and occurred after a mean of 1.34 hours (the differences of these parameters between the dosing groups were not statistically significant). Fifty percent of the dose was recovered in the urine in the first four hours, and plasma levels after 24 hours were undetectable in all but two subjects.

During the Phase III trial in India, it was shown that peak serum level of the drug after intramuscular administration of 11 mg paromomycin base per kilogram body weight was about 20 μg/ml (about 33 μM), and occurred one hour post-injection [Sundar *et al.*, 2007].

4.1.4.5. Trivalent antimony

Trivalent antimony compounds, such as tartar emetic, were used to treat leishmaniasis and schistosomiasis in the early part of 20th century but were associated with significant toxicity and were superseded by safer therapies when these became available.

Trivalent antimony compounds are poorly absorbed after oral administration. They were used orally as emetics and expectorants.

The toxicity of trivalent antimony compounds is probably related to their slower excretion. They were administered intravenously. If injected locally or extravasated they cause severe pain due to tissue damage. They have greater affinity to cell than plasma proteins, and tend to accumulate during treatment,

and are slowly excreted, mainly in the urine, during several months thereafter. Prolonged treatment may lead to subacute poisoning.

Parenteral administration was almost universally associated with electrocardiographic changes, frequently with bradycardia, hypotension or arrhythmias, and occasionally resulted in sudden death.

One study examined the excretion of pentavalent and trivalent antimony preparations in hamsters and human volunteers [Goodwin and Page, 1943]. After an intravenous dose of 50 mg of stibamine glucoside, blood concentration of antimony at one hour post-injection was 1.6 mg Sb^{III}/100 ml (16 mg Sb^{III}/litre, or 16 μg/ml) and 0.8 mg Sb^{III}/100 ml at 3 hours.

4.2. Materials and methods

Recruitment and follow up of patients are described in detail in Chapter 3. In this chapter, the details of parasitological work are described.

4.2.1. Parasite isolation and culture

The Leishmaniasis Control Center of Aleppo kindly provided a separate room for the research laboratory. This room was equipped with locally made incubators (F. Jarad Company, Mhardah, Hama Governorate, Syria) and an air conditioner (this option was cheaper that buying a refrigerated incubator), a desk, a fridge/freezer with a separate freezer compartment and, later, a locally manufactured bespoke class 2 biosafety hood (Hisham Janat, Aleppo, Syria). An inverted microscope (Carl Zeiss, German Democratic Republic) was kindly lent by the Department of Microbiology at Damascus University Faculty of Medicine.

Alcohol burners were purchased and kept in the four study health centres. Ethanol (95%) was purchased from a state pharmacy in one-litre bottles as necessary. The ethanol was used without dilution both to refill the burners and for skin disinfection prior to obtaining biological material from patients.

Undiluted household chlorine bleach (Clorox, Saudi Arabia) was used for disinfecting contaminated tools before discarding them.

Culture media were prepared in batches, as necessary, at the London School of Hygiene and Tropical Medicine (LSHTM) and sent by express mail. Agar slopes supplemented with rabbit blood and penicillin, streptomycin and gentamycin were prepared in Nunclon tubes (Nunc, Denmark), 2 ml in each. M199 (Sigma) [Coderre *et al.*, 1983] (Appendix 7) was distributed into 15-ml centrifuge tubes. Upon receiving the media they were transferred to the refrigerator. NNN agar tubes were kept in the refrigerator and the liquid medium, except one or two tubes, in the freezer. The necessary materials were carried in a bag, with cold boxes during the summer.

The routine protocol for obtaining biological material for analysis, as recommended by the Syrian MoH, was followed. About 2 ml of the liquid medium was added to the agar slope over the burner flame immediately before inoculation, the cap was replaced promptly on both tubes, and the agar tube labelled with patient ID, passage number (the initial inoculation was considered Passage 0) and the date of isolation. The lesion was wiped with a piece of cottonwool soaked in ethanol, left to dry, then scraped with the tip of a sterile disposable lancet blade until the tissue liquid appeared. This drop was touched with a sterile disposable Pasteur pipette (pastette), transferred into the tube, and the cap replaced promptly, all done over the flame. The rest of the biological material was used to prepare a smear, the analysis recommended by MoH.

Until the end of working hours the tubes with cultures were kept at room temperature. On the way back to the lab at LCC the tubes were kept in a bag or, during winter, in an internal pocket.

In the lab the tubes were transferred to a 26°C incubator, situated in the air-conditioned lab. In case of a power cut, upon resuming of power supply the air

conditioner would switch on automatically, so room temperature would not usually exceed 24°C in the hot season.

The tubes were examined under an inverted microscope at 10×6.3 (the maximum available magnification) at least once a week for active promastigotes. Within 2–3 weeks after isolation the samples, whether positive or negative, were subpassaged into the same biphasic medium, unless contaminated. Negative samples were re-examined until they became at least 40 days old, and samples that did not convert to positive during this period, as well as all contaminated samples, were discarded with LCC laboratory waste after every tube was opened and topped up full with either undiluted chlorine bleach or 95% ethanol, then the cap replaced tightly.

4.2.2. Transport and cryopreservation

The cultures that were positive, not contaminated and were subpassaged, were prepared for sending to LSHTM for preservation. The tubes with cultures were wrapped in parafilm, then into a diaper and sent by express mail. It was aimed to send different passages of the same isolate separately (Figure 4.1). The parcels were usually sent on Sunday (a workday in Syria) and were normally delivered to the School Reception on Wednesday or Thursday morning.

In London, the cultures were placed in a refrigerated incubator at 26°C overnight to settle, then transferred to flasks with 10 ml of M199 (Sigma) with 20% heat-inactivated foetal calf serum (HIFCS), and the passage number would increase by one. After achieving an acceptable concentration of active promastigotes in the medium, the cultures were centrifuged, the pellet resuspended in 4 ml of M199 with 20% HIFCS and 8–10% dimethyl sulfoxide (DMSO), distributed to properly labelled 1-ml cryovials, frozen slowly in a –80°C freezer for 24 hours, then transferred to liquid nitrogen. Each isolate was frozen in triplicate or quadruplicate, having a passage number of 1 for original isolates (labelled at isolation "*Passage 0*") or 2 for the isolates subpassaged in Aleppo.

4.2.3. Reviving frozen isolates

Frozen cultures were removed from liquid nitrogen and unfrozen quickly in a 34°C incubator, then each culture was inoculated into two flasks containing 10 ml of M199 with 20% HIFCS. These cultures were marked with the same passage number as the frozen isolate (e.g. *"Passage 1 from liquid nitrogen"*). After achieving successful growth, normally in 4–7 days, the cultures were sub-passaged into larger flasks containing 20 ml of the same medium. One of these flasks was used for sensitivity assays, the other for back-up freezing, and cultures in the small flasks were used for preparing pellets for typing and, if necessary, fingerprinting.

4.2.4. Amastigote–macrophage assay

4.2.4.1. Drugs

The following drugs were used in the assays:

19. Sodium stibogluconate (SSG; Pentostam™, GlaxoSmithKline, UK), kindly supplied by the manufacturer in the form of powder with 31.3% pentavalent antimony content. This preparation has been used as a gold standard for pentavalent antimonials.
20. Glucantime™, commercial formulation (Sanofi-Aventis, France), from batch 711 manufactured October 2004 that was in use in primary health care centres in Aleppo during the study period, kindly provided by Leishmaniasis Control Center of Aleppo. It contains 300 mg of meglumine antimoniate per millilitre with pentavalent antimony content of 81 mg/ml.
21. Amphotericin B (AmB, Fungizone, Bristol-Myers Squibb, UK): shown to be almost uniformly effective against *L. donovani* infection, even at low doses [Davidson and Croft, 1993].
22. Trivalent antimony (Sb^{III}) preparation (potassium antimony(III) oxide tartrate hemihydrate, Fluka Chemika, Buchs, Switzerland): no longer used in the treatment of leishmaniasis. Generally accepted to be the active form of antimony against *Leishmania*: pentavalent antimony is reduced to

trivalent form inside the cells [Goodwin and Page, 1943; Brochu *et al.*, 2003; Mukherjee *et al.*, 2007].

23. Miltefosine (Zentaris, Frankfurt, Germany): the first oral antileishmanial drug with demonstrated efficiency against visceral leishmaniasis in India [Sundar *et al.*, 2002], possible efficacy against cutaneous leishmaniasis caused by *L. braziliensis* in Bolivia [Soto *et al.*, 2008] and a recent report of two cases suggested it might be a promising candidate for the treatment of cutaneous leishmaniasis due to *L. tropica* [Killingley *et al.*, 2009]. A clinical trial comparing miltefosine to Glucantime for *L. tropica* infection is being conducted in Iran [Killingley *et al.*, 2009].
24. Paromomycin (PM; paromomycin sulphate, Sigma-Aldrich Chemie GmbH, Steinheim, Germany): this drug was used for the systemic, local (ointments) or intralesional treatment of leishmaniasis in the Soviet Union [Shuvalova, 2001], and is used in the form of ointments in Israel [Shani-Adir *et al.*, 2005].

4.2.4.2. General description

The protocol used at LSHTM [Yardley *et al.*, 2005; Yardley *et al.*, 2006] for parasite drug sensitivity determination was followed. Certain parameters may differ between *Leishmania* species, so different combinations were tested before the actual experiments began, as described in "Assay optimisation" below.

Female CD1 mice (Charles River Ltd., UK) were injected intraperitoneally with 2 ml of sterile 2% starch solution. After 24 hours the exudate containing macrophages was harvested by peritoneal lavage into cold RPMI-1640 with penicillin and streptomycin. The fluid was withdrawn and dispensed into 50-ml conical centrifuge tube(s). The tubes were kept on ice while in transport. On arrival the suspension was spun down at 1500–2000 rpm for 10 minutes in a centrifuge cooled to 4°C, the supernatant discarded and the pellet resuspended in 10 ml of cold RPMI-1640. Cells were counted in a Neubauer haemocytometer. The suspension was diluted to 5×10^5 macrophages/ml, then distributed into wells mounted on 16-well glass slides (Nunc, Rochester, NY), using Eppendorf

multidosers and syringes, 100 μl in each well (equivalent to 50,000 macrophages per well). This day is considered Day 0 of the experiment.

Eight slides were used for each full assay. The first two slides were controls, with four wells used in each. The other six slides were used for assaying serial dilutions of drugs, each drug in four concentrations, each concentration in quadruplicate (Figure 4.2).

After 48 hours (Day 2) the cell cultures were infected with a suspension of *Leishmania* promastigotes.

After 24-hour incubation, one control slide was stopped: the medium removed, the wells detached, the slide fixed with absolute methanol for one minute and stained with 10% Giemsa solution for ten minutes.

In the other slides the medium was removed using sterile disposable pastettes, then 150 μl of medium was added into each drug assay well and 200 μl into five-day untreated control wells.

Drug solutions were prepared in 24-well tissue culture plates with four serial dilutions of each drug, in four-fold the desired concentrations. 50 μl of solution was added to each well to give the desired concentration in the total 200 μl volume.

On Day 5 the medium was replaced and fresh drug dilutions added, as above, and on Day 7, i.e. after a five-day exposure to the drug, the experiment was stopped as described above for the control slide.

The slides were examined microscopically at ×1000 with immersion. In each well 100 macrophages were counted, and the percentage of infected macrophages was recorded.

The percentage inhibition was calculated according to the following formula:

$$\%inhib = \frac{\mathrm{inf}_{test} - \overline{\mathrm{inf}_{ctrl}}}{\overline{\mathrm{inf}_{ctrl}}} \times 100$$

where:

%inhib is percentage inhibition,

inf_{test} is percentage infection in the test well,

$\overline{inf_{ctrl}}$ is the mean percentage infection in the four 5-day control wells.

The data on percentage inhibition in every well were entered into a Prism (version 4.2 for Windows, GraphPad Software, Inc.) template, and the 50% effective concentration (EC_{50}) was calculated.

4.2.4.3. Assay optimisation

4.2.4.3.1. Parasite-to-macrophage ratio

The assay was conducted using one reference strain of *L. tropica* (MHOM/SU/74/K27, abbreviated here as K27) and two field isolates, A021/p and K100/p. The macrophages were infected in four ratios: 3:1, 5:1, 7:1 and 10:1. Different slides were stopped after 24, 48 and 72 hours and 5 days post-infection. The aim was to obtain an infection level of 70% or more after 24 hours rising to 80% or more after five days.

One of the field strains gave very high infection levels (>91% after 24 hours and >94% after five days) at all parasite-to-macrophage ratios, the other, at 5:1 or more. The reference strain K27 was contaminated, so only the 24-hour slide was available to evaluation. It failed to reach 70% infection level even at the 10:1 ratio. Later on, reduction of parasite-to-macrophage ratios to 3:1 was tried again with different drug concentrations (*see* "Drug concentration range"), resulting in low infection levels in two of the three strains tested.

4.2.4.3.2. Washing

In addition, selected slides were washed with plain cold RPMI-1640 24 or 48 hours post-infection to remove excess promastigotes. Washing did not improve the number of promastigotes in the final slides, so it was not employed in further experiments with one exception: we tried to remove the actively swimming promastigotes that were clearly visible under the inverted microscope before stopping one slide after the end of the experiment. The wells

were repeatedly washed with cold phosphate-buffered solution (PBS) and re-examined under inverted microscope after each wash. Each wash was performed by pushing PBS several times then aspirating and discarding it. The density of promastigotes became lower only after six to seven washes, and after fixing and staining the slide it became obvious that most macrophages were washed away as well.

4.2.4.3.3. Drug concentration range

About twenty field isolates were then tested using standard drug doses with three-fold dilutions, each dose in quadruplicate. The concentrations of antimony preparations were calculated according to antimony content, while for the other drugs molar concentrations of the compound were used.

SSG and Glucantime were used at 80, 27, 9 and 3 μg Sb^{V}/ml; Sb^{III} at 30, 10, 3.3 and 1.1 μg Sb^{III}/ml [Yardley *et al.*, 2006]; AmB, at 2, 0.66, 0.22 and 0.07 μM; miltefosine, 30, 10, 3.3 and 1.1 μM; and paromomycin at 50, 16.7, 5.55 and 1.85 μM.

The microscopical examination revealed that all the isolates tested had EC_{50} values for SSG (and Glucantime) and Sb^{III} higher than the highest drug concentration used (80 and 30 μg/ml, respectively), with high parasite burdens but were all very sensitive to AmB (several isolates showing EC_{50} values below 0.07 μM, the lowest concentration used).

The following experiment used a maximum of 120 μg Sb^{V}/ml against three isolates at two parasite-to-macrophage ratios, 3:1 and 5:1. The top concentration of AmB was reduced to 1 μM. The concentration of Sb^{III} remained unchanged because higher concentrations cause toxicity to macrophages. The results did not change significantly: all the tested isolates had EC_{50} values higher than 120 μg Sb^{V}/ml, and at 3:1 infection ratio only one isolate exceeded 80% infection in untreated control wells. The range of EC_{50} values for AmB lay between the two lowest concentrations for two of the three samples, so in subsequent experiments the maximum dose of AmB was further decreased to 0.5 μM.

The next experiment was designed to test for drug response at Sb^V concentrations up to 500 μg/ml. In order to cover the lower part of the range, five-fold dilutions were used: 500, 100, 20 and 4 μg Sb^V/ml.

4.2.4.3.4. Drug exposure period

In addition, an attempt was made to increase the drug exposure period, so the same experiment was run in triplicate: one arm for five days' exposure (standard duration), and the other two arms for seven and ten days. In the latter two arms the medium was replaced and fresh drugs added on the fifth day of exposure, and in the ten-day arm, also on the seventh day of exposure.

Of the three samples tested one had slight bacterial contamination in the five-day arm which became heavy in the 7- and 10-day arms, hence could not be evaluated, and had an EC_{50} value greater than 500 μg/ml at five days. Another isolate had an EC_{50} value greater than 500 μg Sb^V/ml in all the three arms, and the last one showed EC_{50} values of 114, 224 and 137 μg Sb^V/ml at 5, 7 and 10 days, respectively. It was concluded that, 1) the top concentration of 500 μg/ml might also be too low; and, 2) increasing the duration of the experiment beyond five days is unlikely to provide any additional information.

4.2.4.3.5. Final calibration of Sb^V concentrations

The next experiment was conducted with an additional top concentration of 2500 μg Sb^V/ml (2.5 mg). The five isolates tested in this experiment showed EC_{50} values that ranged between 121 and 239 μg Sb^V/ml for SSG and between 468 and 2288 μg Sb^V/ml for Glucantime, so the new maximum of 2500 μg/ml was considered unnecessary for SSG. Because it seemed that Glucantime had lower activity, for it the top concentration was kept at 2500 and the minimum at 20 μg Sb^V/ml.

4.2.4.3.6. EC_{50} calculations: infected macrophages vs. parasite burden

For five isolates an attempt was made to determine EC_{50} values by counting the number of parasites in each macrophage (parasite burden). The number of

parasites inside 25 infected or uninfected macrophages in each well (4 wells per concentration) were counted. The results differed considerably from the classical method. EC_{50} values obtained by parasite burden were considerably lower than those by proportion of infected macrophages, and the difference ranged between 3.6 and more than 30 times. The results are presented in Table 4.2 (for SSG) and Table 4.3 (for Glucantime). However, in this study, determining drug sensitivity by parasite burden for all isolates was not a feasible option due to time constraints.

In many slides, in addition to intracellular amastigotes, different amounts of promastigotes were present. In this case, only the macrophages that contained any amastigotes were counted as positive (infected). Sometimes the promastigotes would considerably obscure the picture making counting extremely difficult. The macrophage was counted as positive only if at least one amastigote could be discerned among the promastigotes. The macrophages that contained promastigotes only, but no amastigotes, were considered negative.

In one of the experiments two days passed between spreading out the macrophages and infecting them. All the isolates tested in that experiment looked remarkably clear, i.e. without promastigotes. It was decided to infect the macrophages after 48 hours of incubation.

4.2.4.4. Validation: Inter-experiment variability (AmB)

4.2.4.4.1. Experiments with different drug doses

Three isolates were tested for AmB sensitivity twice, using different protocols: the first protocol with maximum dose of 2 μM (high dose) before concentration calibration was finished, and the second, with maximum dose of 500 nM (low dose) that was finally used in all the isolates. The results are shown in Table 4.7.

4.2.4.4.2. Experiments with different isolates from the same patient

For seven patients the results of sensitivity testing for AmB were available from different isolates obtained in the course of treatment with antimonial

drugs, tested in different experiments. Assuming that treatment with antimonials does not affect the sensitivity to AmB, these were compared to assess inter-experiment variability. The last row shows the results for the same isolate (K112/p) tested twice using the same protocol but in two different experiments (Table 4.8).

4.2.4.5. The drug sensitivity assay: the final conditions

All the isolates for which the results will be reported were tested in the conditions described below, with the exception of Table 4.7 that displays the impact of drug concentration range on the sensitivity values obtained. All the clinical isolates were tested within seven passages from clinical isolation.

The procedures for macrophage induction and harvesting remained as described above. The same applies to preparation of cell cultures for the experiments. Briefly, mice were induced with starch, and the macrophages harvested after 24 hours, centrifuged, resuspended in RPMI-1640 plus 10% HIFCS, counted, diluted to 5×10^5 cells/ml, plated out in 16-well cell culture slides and incubated at 34°C in 5% CO_2/air mixture throughout the experiment. This day is the Day 0 of the experiment.

Macrophages were infected after 48 hours (on Day 2) at 5:1 parasite-to-macrophage ratio, incubated for 24 hours and drugged (on Day 3) as described above. The drug concentrations finally used in full experiments with a parasite-to-macrophage ratio of 5:1 are outlined in Table 4.4.

The medium was replaced after two days (on Day 5), and fresh drugs added. After further three days (Day 8) the experiment was stopped, the slides fixed with methanol and stained with Giemsa stain as described above.

4.2.4.5.1. Full and Essential assays

Full assays that included all the tested drugs were applied to the isolates obtained before treatment.

In "essential" experiments only the first three drugs in Table 4.4 — SSG, Glucantime and AmB — were used. These included the follow-up isolates

obtained during the course of treatment and repeated assays of pre-treatment isolates (i.e. the isolates that were tested at low doses of Sb^V in the calibration process using all the drugs). This was done due to time constraints.

Amphotericin B was used as a positive control because it was uniformly effective in clearing the macrophages at the doses tested. Miltefosine and paromomycin were included in the assays to test possible alternatives to Sb^V treatment.

4.2.4.6. Priorities – selection of isolates for testing

Due to the large number of clinical isolates (about 150 from acute leishmaniasis patients and 37 from chronic or relapsed patients), priorities for testing were set. According to the aims of the present study, these were as follows:

1. The strains from patients for whom serial (pre-treatment and follow-up) isolates were available;
2. The strains from patients with successful pre-treatment isolates, if the patients were followed up clinically for at least six weeks;
3. The strains from chronic or relapsed patients.

Due to time restrictions, the isolates from patients for whom follow-up data were incomplete or who did not start treatment were not tested. Only a dozen of isolates from chronic/relapsed patients was tested during assay calibration period with low doses of Sb^V, and these tests were not repeated with high concentrations. Thus, only the isolates from the first two categories were tested.

4.2.5. *Leishmania* typing

4.2.5.1. Typing to species level

The isolates eligible for drug assays (n=40) and seven isolates from chronic or relapsing patients were typed to species level. The typing was performed at LSHTM by Dr. Isabel Mauricio by microsatellite analysis of the ribosomal DNA internal transcribed spacer [Mauricio *et al.*, 2004; Kuhls *et al.*, 2005]. The results

were compared to the profiles of reference strains of *L. donovani* (MHOM/IN/80 /DD8), *L. major* (MHOM/SU/73/5-ASKH), and *L. tropica* (MHOM/SU/1974/K27).

In addition, ten of the above-mentioned isolates were subtyped using multilocus sequence typing (MLST) for five enzymes [Mauricio *et al.*, 2006].

4.2.5.2. Fingerprinting

A selection of clinical isolates was sent to Dr. Israel Cruz of *Instituto de Salud Carlos III* (Madrid, Spain) who kindly agreed to perform fingerprinting. The isolates were selected for typing if they belonged to patients from whom serial isolates were available or if patients were members of one family.

Fingerprinting was performed using digestion with two enzymes, RsaI and HaeIII for kinetoplast DNA (kDNA) and cysteine proteinase B antigen-encoding gene (*cpb*), respectively. These enzymes were shown to give the highest polymorphism [Botilde *et al.*, 2006]. The samples were also fingerprinted using DraIII. The procedures used are outlined in Appendix 8.

4.2.5.3. Sample preparation

Pellets for typing were prepared either from live promastigote cultures or from cryopreserved aliquots. The culture or aliquot was transferred to a 15-ml centrifuge tube and topped up with cold PBS to 13–15 ml, centrifuged at 2000 rpm for 10 minutes, the supernatant discarded, the pellet resuspended in 13–15 ml PBS and centrifuged again as above. The pellets were kept in the freezer at –80°C until typing.

Prior to sending the samples to Spain for fingerprinting, the pellets were thawed, shaken to mobilise them and 1 ml of 70% ethanol added to each. After this, the samples were sent by express mail.

All the samples were of sufficient size to perform the necessary tests.

4.3. Results

4.3.1. Drug sensitivity

Eighty isolates from patients with acute cutaneous leishmaniasis were successfully tested (i.e. with acceptable infection ratios in untreated controls and no or insignificant contamination) with high doses of antimony and one isolate from a patient with chronic lesions. For all these isolates the sensitivity to SSG was determined by percentage of infected macrophages. The results of testing using parasite burden are reported in Table 4.5 but not included in further statistical analyses, as that would mean having two different values for the same isolate.

Sensitivity to Glucantime was determined for sixteen isolates, to amphotericin B, for 67 isolates, and to miltefosine and paromomycin, for eleven isolates. For miltefosine and paromomycin the isolates were selected based on good readability of SSG slides. Most of these readable slides were also sensitive to SSG, so the selection is non-random.

The results of sensitivity testing are summarised in Table 4.6. All the isolates were sensitive to amphotericin B. For all the other drugs, some isolates were insensitive, i.e. showed inhibition of growth of less than 50% at the highest concentration used in the assay for that drug. All the isolates tested for paromomycin were insensitive to the drug ($EC_{50} > 50$ μM). Range, mean and quartile values for the isolates for which the EC_{50} values lay within the range of test concentrations of every drug are given in Table 4.6.

4.3.1.1. Sensitivity to SSG and Glucantime

EC_{50} values for SSG and Glucantime, including the results obtained using parasite burden, were plotted, after converting to common logarithms (log_{10}) of the EC_{50} values (Figure 4.3), and analysed by linear regression. Adjusted $R^2=0.721$, $p=0.0001$, showing a highly significant positive correlation between the sensitivities to these pentavalent antimony drugs.

4.3.1.2. Relationship between sensitivity to SSG and other drugs

No relation between sensitivity to SSG and that to AmB or miltefosine was detected. Sensitivity to SSG was classified as either "sensitive" or "insensitive" by selecting the breakpoints in two ways: the isolates with EC_{50}>500 μg Sb^V/ml *vs.* those with EC_{50}≤500 μg Sb^V/ml and the isolates with sensitivity below or equal to the median value (229 μg Sb^V/ml) and those with EC_{50} higher than the median, including the isolates with EC_{50}>500 μg Sb^V/ml. The median sensitivities to AmB for each of the comparisons were compared by Mann-Whitney test, resulting in p=0.170 in the former comparison and 0.787 in the latter, both not statistically significant.

For miltefosine, the results of only nine isolates were available, all corresponding to the isolates with sensitivities to SSG of ≤500 μg Sb^V/ml, and only one isolate corresponded to the group of sensitivities to SSG above the median, and for this reason the two groups, one of which consisted of only one isolate, could not be compared (Table 4.6).

All the isolates tested for paromomycin gave EC_{50} values beyond the range of test concentrations (Table 4.6).

4.3.1.3. Exposure to antimonial treatment and sensitivity to SSG

Fifty-two serial isolates from 23 patients were available. For the remaining 28 patients one isolate was available from each patient. The isolates were classified to exposed and unexposed groups, the former comprising the isolates obtained before treatment and the latter during the course of treatment.

No significant differences in the median sensitivity to SSG were found between the two groups: p=0.141, Mann-Whitney test, when all the isolates were analysed and p=0.21 when the analysis was restricted to patients with serial samples. Exposure to treatment and nominal sensitivity (below vs. above the median) were cross-tabulated, p=0.11 by Pearson's χ^2 test.

4.3.2. Parasite typing

4.3.2.1. Typing to species level

All the parasites typed to species level belonged to *Leishmania tropica*. Subtyping using MSLT showed that all but one belonged to the common heterozygous group, and one isolate was homozygous.

4.3.2.2. Fingerprinting

Seventy-two isolates from 35 patients were fingerprinted using three restriction enzymes: DraIII, HaeIII and RsaI. According to DraIII, all the isolates belonged to one group. Fingerprinting using HaeIII and RsaI grouped the isolates into 29 schizodemes with perfect agreement between both methods.

Twenty-one isolates were available from fourteen patients belonging to six families.

- As a rule, the isolates from one patient belonged to the same schizodeme (SchD).
- In four families comprising nine patients, the schizodeme was common for each family.
- In the fifth family the grandmother had SchD S14, and grandson, S16 at baseline (the homozygous one, K127/p) and S12 before the 4th dose.
- In the last family (3 members), one member with 2 sequential isolates had SchD S15 sharing it with an unrelated patient; another member, S11; and the third member with pre-treatment isolates from two lesions, S10 and S12.
- Unrelated people might share the same schizodeme, but the sharing always happened within the same health centre catchment area (*see* Chapter 3).

4.3.3. Drug sensitivity and treatment course

Parasite sensitivity was categorised into "sensitive" and "insensitive" in two ways: first, all the parasites that had SSG EC_{50} values within the test concentration range, i.e. up to 500 μg Sb^V/ml, were considered sensitive, and those with values beyond the testing range, insensitive (Table 4.9). Second, all the parasites that had SSG EC_{50} values within the testing range were divided into sensitive and insensitive by the median value, 229 μg Sb^V/ml, and this was taken as a breakpoint, but the sample still contained the isolates that had SSG EC_{50} values beyond the testing range (Table 4.10). The median values for the number of injections, treatment duration and time to cure were compared between the patients whose isolates were "sensitive" and "insensitive" parasites at baseline.

The differences between the groups were not statistically significant in the first comparison (below 500 *vs.* over 500 μg Sb^V/ml). In the second comparison (up to 229 *vs.* >229 μg Sb^V/ml) the patients with less sensitive isolates had significantly less visits and shorter treatment duration. The differences in time to cure were not statistically significant.

4.4. Discussion

The most striking finding was the very low *in vitro* sensitivity of our isolates to SSG. In a similar study performed in Iran [Hadighi *et al.*, 2006], where the causative organisms were *L. major* and *L. tropica*, the maximum EC_{50} value reported was 51 μg Sb^V/ml, which is below the 1st quartile in our dataset. That study differed from ours in the length of drug exposure (six days *vs.* five days) and in much shorter length of incubation of macrophages with parasites (two hours *vs.* 24 hours). The authors used 4:1 parasite-to-macrophage ratio and supplemented the culture medium with 15%, rather than 10%, of foetal calf serum. The authors mentioned that they determined EC_{50} both by percentage of infected macrophages and number of parasites per infected cell but did not

mention either whether the results between the two methods were similar nor which method was used for reporting sensitivity values. It is hard to say with confidence whether these differences in the testing protocol can explain the differences in results. This is the only study linking the *in vitro* sensitivity of field isolates of *L. tropica* with clinical outcome that could be identified.

There was a strong positive correlation between the sensitivities of our strains to Pentostam and Glucantime, which is to be expected, as the two drugs have the same active substance, pentavalent antimony.

All the isolates tested were highly sensitive to AmB and insensitive to paromomycin within the drug concentration range used in this study.

Nine of 11 isolates for which the sensitivity to miltefosine was determined were sensitive to the drug. The same isolates were also sensitive to SSG.

In a study of *L. tropica* drug sensitivity in Iran [Hadighi *et al.*, 2007] eight isolates (four sensitive and four insensitive to Glucantime) were tested for *in vitro* sensitivity to amphotericin B, miltefosine and paromomycin. All the isolates tested were sensitive *in vitro* both to miltefosine (EC_{50}=1 μM) and paromomycin (EC_{50}=5 μM), and two of the isolates that were insensitive to Glucantime had high AmB EC_{50} values (17 and 26 μM).

No correlation was found in our sample between the isolate sensitivity to SSG and that to AmB.

The relationship between the sensitivities to SSG and miltefosine or paromomycin could not be established because the latter was uniformly insensitive and for the former only one isolate with low sensitivity to SSG was available.

A recent study of nineteen clinical isolates of *L. donovani* from areas of high and low antimony sensitivity [Kumar *et al.*, 2009] revealed that the isolates from areas with low antimony sensitivity were less sensitive to amphotericin B and miltefosine *in vitro*, suggesting the potential for development of cross-resistance to these drugs. This study also reported a weak correlation between the

sensitivity of promastigotes and amastigotes to AmB and a strong correlation between the sensitivities of these two parasite stages to miltefosine.

The lack of significant difference between the isolates obtained before or after treatment is in agreement with the results of a similar study of *L. tropica* from Iran [Hadighi *et al.*, 2007], but another group working with New World species (*L. braziliensis, L. guayanensis* and *L. panamensis*) found that several isolates obtained from poorly responding patients during the course of treatment became less sensitive to antimony *in vitro* compared to baseline isolates [Rojas *et al.*, 2006]. These authors used a human promonocytic cell line (U-937) in their assays, 20:1 parasite-to-macrophage ratio and 72-hour drug exposure.

The results of parasite typing showed that all the isolates tested are *L. tropica,* confirming the aetiology of leishmaniasis in Aleppo.

Fingerprinting of selected isolates showed that in most cases the organism isolates from the same patient during the course of treatment is the same, and that the parasites isolated from members of one family usually belong to the same strain, with a few exceptions in both cases. The strain with the same fingerprint may affect unrelated patients but in our selection the patients with the same strain would come from a catchment area of the same PHC centre. This is in accordance with the "patchy" distribution of leishmaniasis due to short flight range of phlebotomines.

We failed to find a meaningful correlation between pre-treatment parasite *in vitro* sensitivity and treatment outcome. Moreover, when the isolates were classified into "sensitive" and "insensitive" using the median sensitivity of the numbers within testing concentration range, it seemed that the patients from whom less sensitive parasites were isolated before treatment had significantly shorter treatment duration and required fewer injections. The studies that addressed the relationship between baseline *Leishmania* sensitivity and treatment outcome are few. In the above-mentioned Iranian study [Hadighi *et*

al., 2006] a strong correlation between pre-treatment parasite sensitivity and clinical outcome was reported: the EC_{50} values for the parasites from responding patients was below 10 μg Sb^V/ml, whereas from non-responders it was over 10 μg Sb^V/ml. But another study, from Peru [Yardley *et al.*, 2006], found no correlation between baseline antimony sensitivity of the clinical isolates and clinical outcome. Definitions of treatment success are not uniform. In the Iranian study the treatment success was complete re-epithelialisation with no relapse within 6 months of follow up. In the latter study two measures were used: 1) initial cure within three months of treatment and 2) complete cure meaning initial cure plus no relapse within twelve months after treatment.

Testing clinical isolates may be associated with methodological uncertainties. Patients may suffer from mixed infections with more than one strain present in the lesion. This is illustrated by the apparent change of parasite fingerprint during the course of treatment in one of our patients. Different strains may have different growth and macrophage infection rates, and these differences need not be the same *in vivo* (i.e. in the lesion, where they may change during the course of treatment) and in the culture medium, which means we cannot be fully sure what strain predominates in the culture which was subpassaged more than once before testing, and whether the initially predominating strain (or the one that was more important in defining the clinical picture) was not lost altogether. A similar problem concerning isolating trypanosomatids from naturally infected wild insects was discussed in a recent review [Podlipaev, 2001].

It is possible to conceive that factors other than parasite sensitivity to drugs are more important in determining the clinical course of the disease. One of these factors could be host immune response, which was not tested in this study.

4.5. Tables

Table 4.1. Summary of articles reporting testing Leishmania sensitivity to Sb^V.

Reference	Location	Species	Form	Host	Ratio	Duration of		Concentration range and steps	Counting	EC_{50} values for amastigotes (µg Sb^V/ml)
						infection	*treatment*	*of Sb^V*		
[Berman *et al.*, 1982; Berman and Gallalee, 1985]	Iran? ref: WR 401 (NIH 173)	*major*	amastigotes from mice	PMC (human) diff. to Mφ in 6d.	7.5:1	4h, washing and drugging	6 days; drugs refreshed on day 3.	2 – 251 µg/ml, 2x (?!)	1. number of amastigotes 2. scintillation of radiolabelled cultures	[mean] 2.8 (1.6-3.6) (3H); 4.1 (2.2-5.3)
[Faraut-Gambarelli *et al.*, 1997]	France	*infantum*	amastigotes	Mouse peritoneal Mφ	~4:1 (30:8)	not stated	7 days; drugs refreshed on days 2 and 5.	7.5, 15, 30, 45, 60 µg/ml MGA	1. number of surviving Leishmania cells in 100 Mφ	[range for ~35 samples] 17-234 (?!)
[Lira *et al.*, 1999]	India	*donovani*	amastigotes	Mouse peritoneal Mφ	~3:1	1h, then washed and incubated 72h before 1st treatment	3 days, drugged once.	Seven concentrations, 2-50 µg/ml SSG	1. % Mφ infected; 2. π/infected Mφ; 3. % killing = total π/100Mφ (treated: untreated)	[mean] responsive 2.4±2.6; unresponsive 7.4±3.7. [Range] 1-13.5. ED90s reported
[Hadighi *et al.*, 2006]	Iran	*tropica*	amastigotes	Mouse Mφ, THP	4:1	2h, washing and drugging	6 days; drug refreshed on day 3.	not stated; published EC_{50} values range 2.2—51 µg/ml MGA	1. % Mφ infected; 2. π/infected Mφ; 3. luciferase in THP	2.2-51 µg/ml
[Rojas *et al.*, 2006]	Colombia	*panamensis, braziliensis, guayanensis*	amastigotes	U-937 (human), diff. to Mφ in 96h	20:1	2h, washing, then 24h and drugging	72h; drug refreshed after 48h.	2–128 µg/ml MGA 2x	%Mφ infected.	0.9 — >128

[1] Not stated; inferred from graph.

Reference	Location	Species	Form	Host	Ratio	Duration of		Concentration range and steps	Counting	EC_{50} values for amastigotes (μg Sb^V/ml)
						infection	*treatment*	*of Sb^V*		
[Azeredo-Coutinho *et al.*, 2007]	Brazil	*braziliensis, guayanensis* (field); *panamensis, tropica, amazonensis, mexicana* (WRAIR)	promastigotes, axenic amastigotes in Mφ	J774A.1 mouse Mφ cell line	1:1	4h, wash and drug	72h, drugged every 24h;	not stated; reported EC_{50} results: promastigotes, 0.8–60 mg/ml; amastigotes, 19–55 μg/ml (*braziliensis* only)	1. promastigotes: Coulter counter 2. amastigotes: number in 50 random Mφ/well (*braziliensis* only)	19–55 μg/ml
[Robledo *et al.*, 1999]	SW Colombia	*panamensis, braziliensis*	amastigotes; promastigotes	U-937	20:1	2h, washing; 24h, then infecting	3, 4 and 6 days, renewing drug every 2 days	standardization: 0.85—8500 μg/ml, 10x; assay: 5.3—170 μg/ml, prob. 2x steps, for 4 days	1. % infected Mφ; 2. mean number of parasites per Mφ (for EC_{50})	<5.3 — >170
[St. George *et al.*, 2006]	unknown	*major, tarentolae*	amastigotes, promastigotes	mouse Mφ	6.25:1	not stated	3 days, drugged once?	50 and 75 μg/ml	1. % infected Mφ; 2. amastigotes /100 Mφ	not stated
[Yardley and Croft, 1997]	unknown	*major*	amastigotes, promastigotes	mouse Mφ	5:1	24h	5 days, renewed on day 3	not stated	% of cells infected	30 and 35 (2 strains tested)

Mφ, macrophage; π, parasite; MGA, meglumine antimoniate; EC_{50}, 50% effective concentration; ED90, 90% effective concentration.

Table 4.2. Comparison between EC_{50} values for sodium stibogluconate obtained by counting the percentage of infected macrophages and those by counting the number of parasites within each macrophage. Confidence intervals (95%) are given in parentheses.

Strain	EC_{50} (macrophages)	EC_{50} (burden)
K127/p	205 (107—325)	6.5 (3.3—13.0)
Y132/p	197 (173—225)	55.3 (28.6—107)
A135/p	2405 (465—12k)	101 (50.9—202)
A062/p'	260 (207—326)	"Does not converge"
Neal-P*	330 (281—387)	81.6 (30.9—215)

* MRHO/SU/59/Neal-P is a reference strain of *L. major*. Passage 2 was used.

Table 4.3. Comparison between EC_{50} values for Glucantime obtained by counting the percentage of infected macrophages and those by counting the number of parasites within each macrophage. Confidence intervals (95%) are given in parentheses.

Strain	EC_{50} (macrophages)	EC_{50} (burden)
K127/p	318 (194—521)	17.06 (4.97—58)
Y132/p	534 (313—909)	98.2 (too wide)
A135/p	496 (282—873)	86.7 (53—141)

Table 4.4. Drug concentrations used in the experiments after calibration.

Drug \ Dose	Unit	Max.	High	Low	Min.
SSG	*µg Sb/ml*	500	100	20	4
Glucantime	*µg Sb/ml*	2500	500	100	20
AmB	*nM*	500	167	55.6	18.5
Sb^{III}	*µg Sb/ml*	30	10	3.3	1.1
Miltefosine	*µM*	30	10	3.3	1.1
Paromomycin	*µM*	50	16.7	5.56	1.85

Table 4.5. Comparison of EC_{50} values (μg Sb^V/ml) obtained by counting the percentage of infected macrophages and by the number of parasites within each macrophage for three isolates.

	SSG		Glucantime	
Isolate ID	*macrophage*	*burden*	*macrophage*	*burden*
K127/p	205	6.5	318	17.1
Y132/p	197	55.3	534	98.2
A135/p	2405	101	496	86.7

Table 4.6. Results of sensitivity testing of clinical isolates.

Drug	n	total	%	mean	median	interquart. range		range	
				±SD		1st	3rd	min.	max.
SSG	32	81	39.5	238±114	229	163	265	28.9	525.9
Gme	15	16	93.8	748±552	547	407	1033	30.3	2288
AmB	67	67	100	67.7±26.9	66.6	52.0	84.9	17.7	149.5
Sb^{III}	1	13	7.7	5.4	5.4	5.4	5.4	5.4	5.4
Milt	9	11	81.8	10.4±4.5	9.7	7.9	14.0	2.6	17.5
PM	0	11	0	>50	>50	>50	>50	>50	>50

SSG, sodium stibogluconate (Pentostam™); Gme, Glucantime™; AmB, amphotericin B; Sb^{III}, trivalent antimony; Milt, miltefosine; PM, paromomycin; n, number of results within the range of concentrations tested; total, total number of isolates for which an attempt has been made to determine EC_{50}; %, (n/total)×100.

Units for SSG and Gme are in μg Sb^V/ml; for AmB, nM; for Sb^{III}, μg Sb^{III}/ml; Milt and PM, mM.

Table 4.7. Results of sensitivity testing of three isolates to AmB (EC_{50}, nM) using two different drug dosage protocols, with a maximum 2 μM (high dose) and a maximum 500 nM (low dose).

Strain ID	Low dose	High dose
H020/p	22.3	144.5
Y036/p	41.1	73.2
K100/p	52.7	22.3

Table 4.8. Inter-experiment variability of sensitivity testing (AmB, , nM).

Patient ID	isolation 1	isolation 2	isolation 3	range (-fold)*
Y036	41.1	27.2		1.51
A080	73.3	52.3		1.40
A082	74.1	65.3	38.3	1.93
A092	34.7	65.3	60.4	1.88
A094	53.2	52.7		1.01
Y105	63.7	94.4		1.48
K112	86.6	104.5		1.21

* The range is the maximum EC_{50} result in a series divided by the minimum result in the same series.

Table 4.9. Relationship between baseline parasite *in vitro* sensitivity to SSG (breakpoint at maximum testing concentration) and the number of injections (visits), treatment duration (weeks) and time to cure (weeks).

	up to 500 μg Sb^V/ml			>500 μg Sb^V/ml			p*
	n	mean (SD)	median	n	mean (SD)	median	
Visits	17	12.9 (4.3)	13	30	11.2 (4.3)	12	0.171
Treatment duration	17	21.0 (6.4)	22	30	16.6 (9.0)	15.5	0.106
Time to cure	11	21.9 (7.5)	23	23	22.7 (11.2)	23	0.868

* Mann-Whitney test. SD, standard deviation.

Table 4.10. Relationship between baseline parasite *in vitro* sensitivity to SSG (breakpoint at the median sensitivity within testing concentrations) and the number of injections (visits), treatment duration (weeks) and time to cure (weeks).

	up to 229 μg Sb^V/ml			>229 μg Sb^V/ml			p*
	n	mean (SD)	median	n	mean (SD)	median	
Visits	11	14.7 (3.5)	15	36	11.0 (4.2)	10	0.01
Treatment duration	11	22.7 (6.4)	24	36	16.8 (8.5)	17.5	0.044
Time to cure	7	23.1 (7.9)	23	27	22.2 (10.7)	23	0.87

* Mann-Whitney test. SD, standard deviation.

4.6. Figures

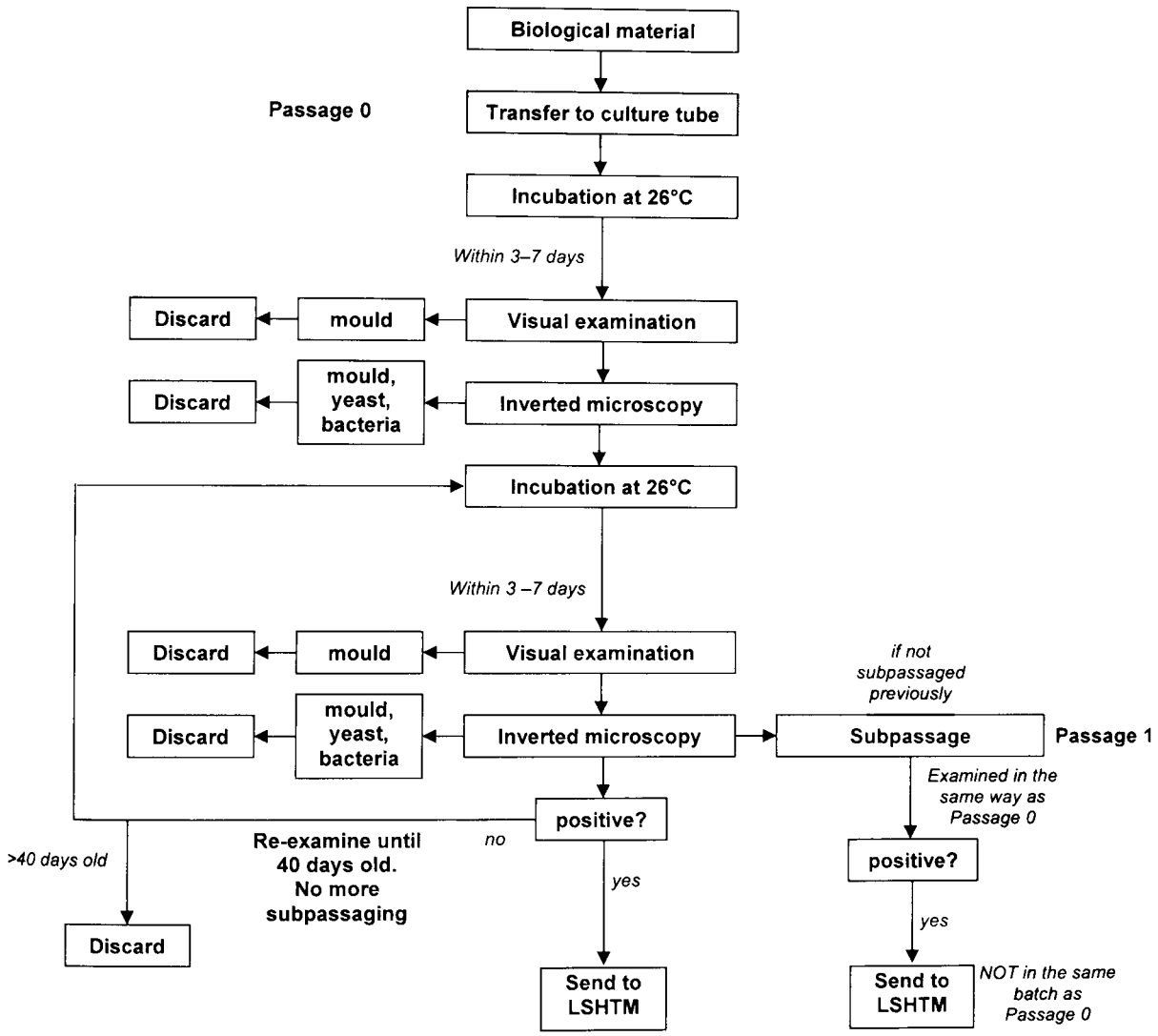


Figure 4.1. Flow chart showing the process of isolation of clinical samples until sending to LSHTM.

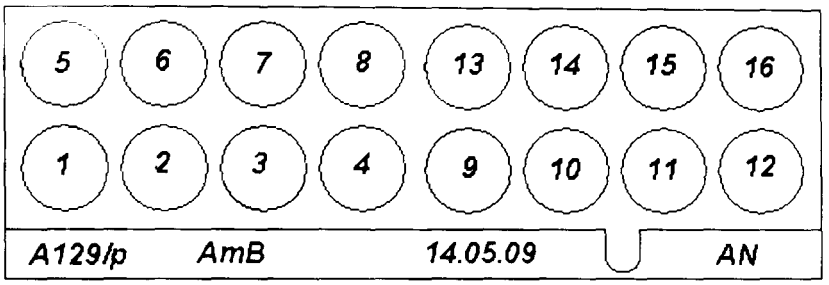


Figure 4.2. Schematic representation of a 16-well slide layout. For untreated controls only wells 1–4 were used. For drug assays the slides 1–4 contained the lowest concentration of a drug, slides 5–8, the next, 9–12 the next higher and 13–16 the maximum concentration. Each slide was marked (from left to right) with isolate ID, drug abbreviation, date of experiment and experimenter's initials. In experiments with more than one arm, additional information, such as parasite-to-macrophage ratio or days of exposure, was added.

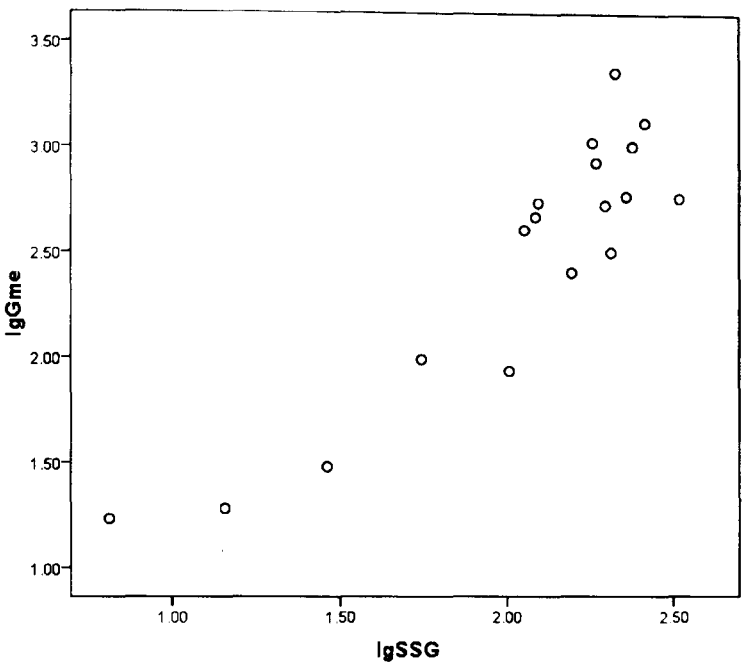


Figure 4.3. Comparison of logarithms of EC_{50} values of SSG and Glucantime The analysis includes the results of parasite burden testing.

Chapter 5. Knowledge, attitudes and practices

5.1. Introduction

Cutaneous leishmaniasis (CL) has been endemic in Aleppo, Syria for centuries, giving the disease one of its common names, Aleppo boil [Canaan, 1929; Hovnanian *et al.*, 1937]. Aleppo is also the site of the first recognisable modern description of CL [Russell, 1756], although the disease may have been described as early as in the ninth century under the name of Balkh sore [Elgood, 1934].

Russell mentioned that the disease is known locally as *habt il senne*, translated by him as *botch of a year*. Peter Borovsky, the author of the first description of the *Leishmania* parasite that is in agreement with current knowledge about its morphology and relationship to host tissues, suggested that the aetiology of similar skin conditions, including those encountered in Aleppo and Biskra, should be investigated as there might be links to the same causative agent [Borovsky, 1898].

Russell's observations suggest that CL has been recognised by the lay population since at least the middle of eighteenth century. However, to our knowledge, no studies were published about the perceptions of the population of Aleppo concerning this common condition. Moreover, it seems there were no publications about popular perceptions of CL in the Old World until quite recently, although statements regarding the perceptions of local people of the disease occasionally appeared in articles dedicated to the epidemiology or treatment of leishmaniasis. However, these statements are probably based on authors' impressions, because no further references are given. Siage, working in Damascus where the causative agent was probably *L. major*, mentioned that the villagers tended to ignore the lesions because they are indolent, and "the scars

are not usually feared of" [Siage, 1964]. A similar statement was made about the perception of CL in the Arabian Peninsula, that this disease "was simply accepted as a fact of life" [Peters, 1988]. By contrast, in a report from Aleppo, Ashford *et al.* state: "Infection is most important as a public health problem in teenagers and young adults, for whom the cosmetic effects are often considered very serious" [Ashford *et al.*, 1993]. These findings are echoed by studies performed in Afghanistan which often report that CL is considered stigmatising, and that people with the disease may face social ostracism. In Kabul girls with scars are considered unsuitable for marriage, and it is considered inappropriate for women with active lesions to raise children, cook or wash for the family [Reithinger *et al.*, 2003; Reyburn *et al.*, 2003]. A later Reithinger *et al.* involved a series of focus-group discussions which confirmed their previous observations of popular perceptions of CL in Kabul [Reithinger, Aadil *et al.*, 2005]. Recently, a more in-depth study was performed in Istalif district of Kabul province, located 50 km to the north of Kabul and inhabited by Tajiks, with anecdotal reports of more than 50% prevalence of leishmaniasis [Stewart and Brieger, 2009]. The authors reported that the local name of the disease, *saldana*, means "one-year sore" (similar in meaning to *habt il senne* in Aleppo, *salek* in Iran and *godovik* in Russia) and confirmed its stigmatising perceptions among the local population.

In Turkey, one study examined the psychological impact of CL in Sanliurfa, a region adjacent to Aleppo governorate [Yanık *et al.*, 2004]. The authors compared active CL patients with healthy controls and healed leishmaniasis patients, and found that active and healed patients showed higher anxiety and depression scores and lower body image satisfaction scores compared to controls. They also speculated about possible stigmatisation but did so in the context of established relationships between conspicuous skin conditions and psychological disorders, i.e. not specifically related to CL. This seems to be the

first and, to date, the only study that addressed the psychological impact of Old-World CL.

An indirect indication of the dearth of published research about perceptions concerning CL in the Old World is the total absence of references to any social research publications about CL in the above-mentioned studies, and, moreover, none of these studies cross-referenced any other of the studies discussed above.

It is worth mentioning that all these studies were performed in the areas where the causative organism is believed to be *L. tropica* and the cycle of transmission is anthroponotic.

5.2. Materials and methods

To provide information on the perception about CL held by the participants in this study a cross-sectional survey of the knowledge, attitudes and practices (KAP) with regard to cutaneous leishmaniasis in Aleppo was undertaken using structured questionnaires.

The recruitment of the patients for the clinical study is described in detail in Chapter 3. Briefly, to be eligible, the patients who referred to one of the four primary health care (PHC) centres where this study was conducted should have leishmaniasis confirmed either by smear or culture, be resident in the PHC centre catchment area (hence likely to continue treatment in the same centre) and have no more than five active lesions. Informed consent to participate in the study was obtained from the patient or patient's caretakers when the patient's age was younger than 14 years.

Each of these four PHC centres was visited on a fixed weekday by the same investigator to enable the follow-up of the patients who would turn up once a week to have their lesions evaluated and injected.

A two-part structured questionnaire with open-ended questions was administered by the principal researcher to the patients or, when the patient was younger than 14 years, to an accompanying elder. The first part of the questionnaire was normally administered on the day of recruitment, frequently

before the eligibility for the clinical study was established. Because the formal establishment of the diagnosis is not likely to affect people's knowledge about and attitudes towards the disease, all the available questionnaires were analysed, regardless of whether or not the patient was actually included in the clinical study. The unit of this study is questionnaire, also referred to by the words "respondent" and "case".

The questionnaire was custom-designed for the purposes of the current study. A copy and English translation is included in Appendixes 9 and 10.

In addition to the data necessary to identify the patient, the questionnaire had two parts. The first part of the questionnaire was administered on recruitment while the second part was filled in at a second interview that took place near or at the end of the treatment course. The first interview consisted of several groups of questions (sections). In the first section, issues related to knowledge of disease name(s), aetiology, transmission, prevention, clinical course and acquired immunity (whether the disease can affect the same person more than once). The second section consisted of questions relating to disease recognition, treatment-seeking behaviour and access to treatment. The third section explored respondent's expectations regarding the treatment: the nature of treatment, how long it lasts and recognition of cure. The fourth section concerned the respondent's attitude to the possibility and necessity of prevention and treatment and what disease manifestations are perceived as the most distressing, while the last section in this part of the questionnaire was related to common practices: sleeping outdoors, using bednets, using insecticides.

The second part of the questionnaire, administered at the second interview when the respondents had been through the treatment process, contained a series of questions related to perceptions of quality of care. Questions were asked about travel time and means to the PHC centre, waiting times within the centre and perceived quality of care at the PHC centre. The questions about

quality of care were administered in a location such that the health centre staff were unable to listen to the conversation.

Open-ended questions were asked in a manner to avoid, as far as possible, suggesting answers. For example, the questions were asked in the form "What is the cause of this disease?", "How do people contract this disease?" not "Can the disease be transmitted by water, insects, etc.?", i.e. without suggesting possible agents other than the ones mentioned by the respondent.

When several children from one family presented together, one questionnaire was filled for them all based on the responses of the accompanying adult. If more than one patient from the same family were adults, we aimed to interview all the adults with a separate questionnaire for each. Thus, one questionnaire might correspond to one or more patients.

It was not always possible to administer the questionnaires to new patients. In many instances the number of patients in the leishmaniasis clinic was high, and we had to follow up the patients recruited earlier without causing them unnecessary delays. The mean number of questionnaires administered in one day was 1.9, the median, 1.5, and it never exceeded five. The time necessary to administer the first part of a questionnaire was usually between ten and fifteen minutes. For the second part it would not exceed five minutes.

While there were some refusals to participate in the clinical trial, of the people who consented to participate no one refused the KAP part of this study. Moreover, one patient who consented to participate in the study but withdrew after consulting her husband stated that her demographic, clinical and social questionnaire data collected on her first visit might still be used.

The responses were coded and entered into Microsoft Excel spreadsheet (Microsoft Office Professional Edition 2003). The spreadsheet was imported into SPSS ver. 16 and analysed.

5.3. Results

5.3.1. Sample description

Seventy part one questionnaires (the component relating to knowledge, attitudes and practices) were available for analysis. Of the seventy respondents who completed the first part of the questionnaire, twenty-six were available for the follow-up interview and completed the second part (health care accessibility and quality assessment) of the questionnaire. The main reasons for drop-out were missing the respondent during the last visit, failure to turn up for post-cure follow-up and drop-out from treatment before cure.

The term respondents relates to the patients above 14 years (who answered the questionnaire themselves) and the caretakers of children under 14 years of age who were suffering from CL. That is, the demographic data relating to age and education, as well as the data on views and behaviour relate to the adults who responded to the questionnaire (patients and caretakers) and do not include demographic data about the children in their charge. In 31 cases the respondents were the caretakers of children with the disease whose age was less than 14 years. In 23 (32.9% of all respondents) of these cases the respondent was the patient's mother (nearly three-quarters of caretaker-respondents), in seven (10% of all respondents), the patient's father, and in one case, the patient's grandmother.

The mean age of the seventy respondents (that is, the age of those who answered the questionnaire – not including the ages of the children for whom the caregivers were responding) was 32.3±12.4 years, the median, 32 years, interquartile range, 22–40.25. Forty-eight (58.6%) of the respondents were female. In 39 cases (55.7%) the respondent was the patient while in 31 cases the respondents were the caretakers of children with the disease whose age was less than 14 years: in 23 (32.9% of all respondents) patient's mother (nearly

three-quarters of caretaker-respondents), in seven (10% of all respondents), patient's father, in one case, patient's grandmother.

The respondents with cutaneous leishmaniasis (patient-respondents) tended to be younger (mean, 30.3, median, 28 years) than caretaker-respondents, i.e. the respondents who did not have cutaneous leishmaniasis themselves but were accompanying a minor patient (mean, 35.2, median, 35 years, Mann-Whitney test, 0.039). There was no difference in sex distribution, years in education, treatment seeking delay or responses related to knowledge or quality of care appraisal between these two groups.

Data on education were available for sixty-seven respondents. Education was recorded as 'years in study', with the only qualitative value for encoding 'university degree'.

About one-third of the respondents, that is, either the patients themselves or the adult reporting on behalf of a child (22 respondents), reported six classes of education (mode, i.e. the most frequently occurring value). Six classes are termed *primary education* in Syria, and until recently this was the minimum obligatory education. A few years ago the minimum was raised to nine classes, formerly termed *preparatory*, now *basic education*. The mean education level was 6.4±4.4 years, median, six, interquartile range, 5–8.

Thirteen respondents (19%) were illiterate, sixteen (24%) had nine years' education or higher, nine finished secondary school (twelve classes) and three obtained a university degree. The remaining six respondents were either university students or had post-school education below university degree.

5.3.2. Knowledge

5.3.2.1. Disease name

The various names given to the condition by the respondents in the survey are presented in Table 5.1. The majority of respondents reported "one-year sore" as the only name for the disease. Two very close variants were

encountered: *habt es-saneh* and *habbayet es-saneh*. Three respondents reported *Leishmania* as the only name, and six said it was a "common pimple", i.e. they did not regard it as a specific condition and sought treatment for a pimple that persists too long. Four respondents reported more than one name: one gave the answers *one-year sore* and *Aleppo sore*, one *Aleppo sore* and *Leishmania*, one *one-year sore* and *Leishmania* and one all three names. The respondent who reported three names had a university degree, and the two other respondents who mentioned *Aleppo sore* had fourteen years of education. This suggests that the name *Aleppo sore* may only be used in Aleppo by more educated people.

However, the differences by sex or educational level (up to six classes *vs.* seven years or more) were minor. The only statistically significant difference was for the name *one-year sore* by educational level when responses were categorised to *one-year sore* and *other* (Pearson's χ^2=0.042).

5.3.2.2. Contagiousness, sources and mode of transmission

Responses about disease contagiousness, i.e. direct person-to-person transmission were available from fifty-seven respondents. Of these, thirty-nine (68%) replied it was not contagious, ten (18%) replied it was, and eight (14%) said they did not know.

In reply to the question about the sources of the disease, many of the respondents mentioned more than one source.

Insects were the most common source mentioned (fifty-five respondents). Forty-eight of these respondents suggested insect bites as the mechanism of causing the disease. The insects mentioned were *baqq* or *barghash* (the local names usually applied to tiny biting flying insects, such as sandflies) in thirty-one cases, five of which also mentioned flies as the causative agent; mosquitoes were implicated by thirteen respondents, four of these also implicated flies and one sandflies; five respondents named unspecified insects and four, flies only. Forty-eight respondents said the disease was caused by insect bites and six said

the insects transmitted the disease from polluted sources, such as polluted water or wells (three responses) and "dirt" (two respondents).

The next reported source of disease was man (twelve responses). Of these twelve respondents seven believed the disease could be transmitted by direct contact (three respondents) or indirect transmission by fomites, i.e. sharing objects such as towels (two respondents). One respondent believed the disease can be transmitted by contact with an infected person or via fomites or transmitted mechanically by unspecified insects. Four respondents who did not believe the disease was contagious said it was vector-transmitted: one implicated sandflies, one mosquitoes, one mosquitoes and flies, and one mosquitoes, flies and sandflies.

Twelve respondents reported microbes as cause of the disease, and one of them named *Leishmania* as the causative agent. Four respondents said the microbe was transmitted by an insect bite, two (including the above-mentioned who knew the name of *Leishmania*) believed the transmission is mechanical, one said the sandfly injects microbes into blood, one suggested air-borne transmission, and one named contact with dirt.

Eight respondents said the disease comes from dirt or dust, either by insects (three respondents) or direct contact (two respondents). The rest did not explain the transmission mode.

Water as the cause/source of disease was mentioned by seven respondents. Three believed the disease resulted from drinking water, two said it was transmitted by insects. The other two respondents suggested swimming or washing, i.e. direct contact.

Three respondents believed the disease resulted from a trauma (a splinter, any skin trauma or scratching an itch, one response each) and one believed it was related to psychological distress.

The responses were ranked by frequency and differences were analysed by sex and education. Education was categorised as primary (up to six classes in

school, inclusive) and more than six classes. The first four ranks are presented in Table 5.3. In all groups *baqq* (i.e. sandflies) were mentioned most frequently as the cause of the disease.

No significant differences were noted between patient-respondents (CL patients themselves) and caretaker-respondents (who accompanied CL patients).

5.3.2.3. Prevention, recurrence and time to healing

Forty-one of sixty-six respondents (62%) believed the disease could be prevented, nine (14%) believed it could not and sixteen (24%) said they did not know. Of sixty-five respondents, forty-two (65%) believed the disease might affect the same individual more than once, twelve (18%) believed the disease never recurred and eleven (17%) did not know.

The respondents suggested several prevention modes. Each respondent might give more than one reply to this question. The replies were available from 44 respondents (11 males, 33 females; 18 with education of up to six years, 16 with education of more than six years). The most common modes of prevention are ranked in Table 5.4. Bednets, followed by insecticides, personal hygiene and waste evacuation were the most common responses in the whole sample, among females and those with more than six years in education. Respondents with up to six years in education differed from the complete sample in placing the insecticides in the first rank and the bednets in the second. Among males, the bednets did not fall among the first four choices, and this difference in choosing bednets was the only one that had a marginal statistical significance between sexes ($p=0.049$, Pearson's χ^2).

Eight respondents suggested both bednets and insecticides, while all the other combinations of more than one response were unique.

Responses about time to healing were available from fifty-eight respondents. The mean time to healing from the forty-two respondents who gave finite periods was 11.25±6.3 months, the median, 12 months, interquartile

range, 6–12.75 months. The responses ranged between one month and two years, and twenty respondents (48%) said the lesion needed one year to heal.

Thirteen respondents said they did not know. Three respondents stated the disease does not heal unless treated.

5.3.3. Disease recognition and treatment seeking

The respondents were asked who had first noticed the appearance of a lesion. The lesions were noted by the patient in 37 cases (53%), followed by the patient's mother in 25 cases (36%), for a total of 89%. The other responses were parents and relatives (three cases each) and daughters and father (in one case each).

In the patient-respondent group the lesion was noted by the patients themselves in 85% of cases, whereas in the caretaker-respondent group they were noticed by others in 87%.

The most common descriptions given to the eruption were a small red papule. The word used was *habba*, literally "a grain", commonly used to describe well-delineated raised closed skin eruptions. The eruption was described as papule by fifty respondents, followed by insect bite by ten. Fourteen respondents described its colour as red (of the twenty-one who mentioned the colour). Nine said it was small, seven noticed slow growth and six said it did not change.

Responses about measures taken after the lesion was noted were using an antiseptic (described as *spirto*, a common reference to ethanol) in nineteen cases, doing nothing about the lesion in sixteen and applying a cream or ointment in thirteen cases.

Forty-three respondents did not report receiving any advice for home management of their lesions. Of those who received any advice, the most common, in seven cases, was to refer to a primary health care centre. All the other responses were unique.

Advice to seek treatment (*"Who told you that your condition requires treatment?"*) was given by mothers in 21 case, decided by the patient without advice from others in 14 cases, family in 12 cases, fathers in six cases and neighbours in four cases.

Treatment-seeking delay ranged between immediate to one year. The mean delay was 2.4±2.1 months, the median, 2 months, interquartile range, 1–3 months.

The patients were referred to the health centre (*"Who told you to go to this health centre?"*) by mothers in 23 cases, knew the centre themselves in 20 cases, or by fathers or relatives in six cases each.

In thirty cases the decision to treat the disease was taken by the patient, in 24 cases by mothers and in twelve cases by fathers.

In thirty cases the patients were accompanied on their first visit by their mothers, in 26 cases they came alone and in six cases accompanied by fathers.

This was reflected by the differences in replies between the cases where the respondents were patients themselves (patient respondents) and caretaker respondents: the patient respondents were more likely to come unaccompanied (in two-thirds of cases) whereas the caretakers, usually mothers, were accompanying younger patients in all cases. The decision to seek treatment and referral to health centre was by the patient in the patient-respondent group (in three-quarters and half of the cases, respectively) and by mothers in most cases in caretaker-respondent group.

5.3.4. Expectations

Replies about what the respondents knew about and expected from the treatment were available from 67 respondents.

5.3.4.1. Nature of treatment

Four respondents did not know what the nature of treatment was. The rest said it was injections. Of these, 54 said the injections are made into the lesions,

three, that the disease can be treated with intralesional or intramuscular injections, and six only knew it was injections, but not the mode of administration.

5.3.4.2. Treatment duration

The expectations, as reported by the forty-nine respondents who gave quantitative responses, ranged between three weeks and one year. The mean expected treatment duration was 3.2±3.3 months, the median, two months, interquartile range, 1–4.5 months. Ten respondents reported having no idea about the length of treatment duration, and eight replied it could not be predicted because it depended on the clinical course of the disease.

5.3.4.3. Cure

The question asked was "How do you know you are cured?" Most respondents replied the lesions would disappear (29 replies) or diminish (15 replies). Twenty-one respondents said the cure is determined by health centre staff, and two replied they did not know. However, the proportion of those who continued their treatment until formal dismissal was the same among those who said cure was to be determined by the health centre staff and those who suggested their own criteria (31% *vs.* 32%, p=0.936, Pearson's χ^2).

5.3.5. Attitudes

5.3.5.1. Prevention

The respondents were asked "Should the people try to prevent this disease?" "What needs to be done to prevent the spread of the disease?". Sixty-seven responses were available for the first and fifty for the second question.

Fifty-four respondents believed people should try to prevent the disease, ten replied these attempts were worthless and two did not know how to answer.

The suggested modes of prevention were insecticides (25 responses), bednets (14 responses), public waste evacuation (13 responses), personal hygiene (10 responses), safe water supply (7 responses) and window mesh (6 responses). The same trend was observed in both education level groups and in both sexes with one exception: males placed window mesh, rather than bednets, in the second rank.

The respondents might give more than one reply. The most common combinations were bednets and insecticides (5 cases) and window mesh and insecticides (3 cases).

5.3.5.2. Treatment

All the respondents believed one must seek treatment for the disease. This unanimity is natural as all the respondents were interviewed when they actually sought treatment.

The most frequent reason for seeking treatment was to prevent mutilation (23 cases) or the development of a deep ulcer (6 cases), for a total of 29 replies. The next most frequent reply was to speed cure and avoid multiplication of lesions (9 replies for each), or avoid lesion growth (8 cases).

To the question what happens if the disease is not treated, eighteen respondents replied the lesion would ulcerate, thirteen said it would grow and eleven that it would spread (multiply in number and appear in other locations) or leave permanent marks.

When naming the most unpleasant manifestations of the disease, most respondents mentioned the appearance of the lesion (48 cases), followed by the permanent mark it is known to leave (23 cases). Both these points were mentioned together in nine cases. Other replies were encountered in three or less cases each and included itch, pain, oozing or the feeling the lesion is unclean.

5.3.6. Practices

Questions about sleeping outdoors and using bednets and insecticides were asked to elucidate the common practices. The replies were available from 68 respondents.

Forty-two respondents reported sleeping outdoors during the summer. Twenty-two reported using bednets, plus seven said they used them "sometimes". Twenty-four respondents reported using insecticides, one, insect repellent tabs and nine occasional use of insecticides.

5.3.7. Quality of care appraisal

Towards the end of the therapy process a second questionnaire was administered to ascertain the participants' views on the ease of accessing treatment and the quality of care provided. These interviews took place, on average, seven months after the first questionnaire has been administered. The questions about the quality of care included travel time to the health centre and the mode of transport, waiting time within the health centre, the perceived attitudes of the health centre staff and an overall qualitative evaluation of service as *very bad, bad, acceptable, good, very good,* encoded with numbers 1 through 5. These questions were asked near or after the end of therapy, preferably in such a way that the staff were not able to listen to the conversation. The patients who dropped out and could be contacted were also asked about the reasons of interruption of treatment.

The major limiting factor in the interpretation the results from the second round of questionnaires is that only 26 respondents were available to fill the exit part of the questionnaire.

Replies about travel time were available from 26 respondents. Nineteen of these (79%) reported travel time of fifteen minutes or shorter, and nine of them estimated travel time to be ten minutes. Another two respondents failed to provide an estimate, just characterised the travel time as "short".

The replies about mode of transportation were available from eighteen respondents. Nine of these arrived on foot and three in a public minivan. A taxi or a private car was used by two respondents each, and a bicycle or a motorcycle, one respondent each.

The replies about waiting time within the health centre were available from 26 respondents, and ten of these replied only with qualitative estimates: long (3 cases), medium (one case) or short (6 cases). Among the remaining sixteen respondents who gave quantitative replies, the median waiting time was ten minutes. Four respondents said they did not have to wait at all but waiting times ranging between 45 minutes and up to 2½ hours were also reported in five cases. These prolonged waiting times (and two of three reports of "long" waiting times) were associated with one urban and one rural health centre. In the former large crowds formed after the reinforcement of health district system when all the coming patients needed to formally register in the health centre (see *Structure of relevant health services* in Chapter 1). In the latter centre cutaneous leishmaniasis was treated on one weekday only, and on that weekday during the peak season more than one hundred patients had to be treated.

The quality of service was appraised by 25 respondents, eleven of them were from one urban centre, and the rest distributed evenly between the other three centres (4 or 5 cases each). Thirteen of the respondents estimated the quality of care as *good*, four as *very good* and three as *acceptable*. *Bad* and *very bad* responses were received in two and three cases, respectively. Two of lower-than-acceptable appraisals were associated with the crowds at one of the urban health centres due to health district policy (and lead to drop-out) and the other three related to perceived staff arrogance and lack of care in the other urban and one rural centre.

When we compared the respondents who did not complete follow-up until complete cure to those who were followed up until cure was documented

(Table 5.5), the responses of the former (ten respondents) distributed more or less evenly between *very bad* and *good*, but none characterised the services as *very good*. On the other hand, all the fifteen respondents with completed follow up characterised the services as *good* or *very good*. The median scores, with 1 corresponding to *very bad* and 5 to *very good*, were 2.5 in the former group and 4 in the latter, and this difference was statistically significant ($p<0.001$, Mann-Whitney U test).

Only six drop-outs of the forty-three people not known to have achieved cure in this sample could be contacted. Two of them (mentioned above, siblings) left because the service was perceived as awful, one reported lack of response, one considered the lesion improved enough (and was afraid of injections), one of them was a university student and could not visit the health centre during its opening hours, and one moved away from Aleppo for work (as reported by his mother on the telephone). These people also replied to the questions from the second part of the questionnaire.

5.4. Discussion

To our knowledge, this is the first attempt to describe knowledge, attitudes, expectations and practices of people in Aleppo, Syria, with regard to cutaneous leishmaniasis, a common condition in that area.

We show that the disease is widely recognised by the participants in this study and is referred to by its traditional name, *habbt il senne*, or "one-year pimple", first documented more than 250 years ago [Russell, 1856]. This name is almost identical to the one reported from Afghanistan, *saldana* [Stewart and Brieger, 2009], *salek* in Iran [Elgood, 1934] and from Russia, *godovik* [Borovsky, 1898], all of which incorporate *year* in their meaning.

Most respondents mentioned insects as factors of the disease: either direct cause or vector. Forty-four percent of the respondents implicated sandflies as the cause of the disease, and nearly one-quarter implicated mosquitoes. *Sheikh saket*, or "silent old man", that was used in a health education brochure as the

popular name for sandflies in Aleppo, was not mentioned by any of our respondents. However, we did not ask the respondents to describe the insect they mentioned. The next common response for disease source was man, followed by "dirt" and water. This corresponds well with the results of the Afghan study where the insects, sandflies and mosquitoes, were perceived as the most common cause, followed by contagion, then environmental factors. In that same study, about half of the respondents implicated sandflies but only ten of the participants mentioned the bites as the mode of transmission [Stewart and Brieger, 2009], compared with 47 of 52 in our sample. The authors did not mention the exact number of their respondents, only mentioning they recruited "eight groups <...> of six to nine individuals each", which is more or less comparable to our sample size.

In the study of Stewart and Brieger (2009) several options of home treatment for cutaneous leishmaniasis are described. This cannot be compared to our study because in Aleppo the home treatments tended to be used prior to disease recognition, while the patients still believed the lesion was a "common pimple", and after leishmaniasis was suspected the treatment was usually sought in the local primary health care centre. This may be due to better accessibility of medical care in Aleppo than in Istalif, where the clinic was not easily accessible for the majority of the local population and did not offer free diagnosis and treatment, better knowledge of the people in Aleppo about the disease and, probably, the effectiveness of health education in Aleppo. However, the sample of this study were people who were already accessing to PHC and therefore, their perceptions and reported actions may not be representative of the total population of leishmaniasis sufferers.

With regard to prevention measures, our respondents placed standard bednets in the first place, insecticide spraying in the second and personal hygiene in the third. The people with a lower educational level placed insecticides before bednets, and males had a completely different ranking with

personal hygiene in the first place (Table 5.4). In the study by Stewart and Brieger (2009) the bednets and insecticides were also the first two options, with the former preferred by females and the latter by males. However, in our study the responses related to the way the question was posed. When a similar question was asked in the Attitudes section, phrased as "What needs to be done to prevent the spread of the disease?" rather than "How can one prevent this disease" in the Knowledge section, the insecticides came first, followed by bednets and public waste evacuation. This is because in the Attitudes section the replies contained not only personal measures but also those that should be taken by public authorities.

The most unpleasant reported manifestation of cutaneous leishmaniasis in our sample was the appearance of the lesion and fear of permanent mark.

We show that health centres in Aleppo are easily accessible to most patients in our sample, with travel time of less than fifteen minutes in about 80% of cases. Half of the respondents arrived on foot. Waiting time was also short in most cases.

Twenty of 25 respondents rated the quality of care as "acceptable" or better.

Our data show that for the respondents in our sample the barriers to treatment seeking are generally low. The disease was recognised by the patients or their relatives in most cases, health centre locations are known, and free diagnosis and treatment is offered.

Our study has several limitations. First, our respondents were recruited in the health centres where they came to seek diagnosis and treatment of leishmaniasis. For this reason, it is not surprising they all believed leishmaniasis should be treated, since those who believed it should not were far less likely to be encountered by us. Thus, our sample is not representative of the entire population at least in this point.

We did not ask the respondents to describe the insects that cause or transmit the disease but only recorded the reported insect designation. We also did not

ask specific questions about possible marginalisation, although, most probably, if any significant social oppression existed, it would have been mentioned in one way or another. We also did not explore the signs or symptoms which necessitate treatment seeking.

The data on travel and waiting time, and on perceived quality of care in our sample may be further biased due to high drop-out rate. The drop-outs could be contacted in a minority of cases only so the conclusions from their responses can only be regarded as provisional but it can be inferred with some confidence that patient compliance is probably related to perceived quality of service, given the fact that none of the patients who did not complete the treatment rated the services as *very good* and, on the other hand, all the patients who did complete the treatment rated them as *good* or *very good* (Table 5.5). Only one of our six respondents explicitly stated he was afraid of injections, and one could not come during working hours of the PHC centre. It also seems that the attitude of our respondents relating to cure recognition (by health-centre staff *vs.* improvement of the lesion as assessed by the patient) was not predictive of treatment completion until cure.

Two of lower-than-acceptable appraisals were related to disruptions in health centre operations because of sudden enforcement of a new policy and a three-week measles vaccination campaign when little or no other services were provided.

It cannot be inferred from our PHC-based sample how accessible CL diagnosis and treatment services are for the entire population in terms of travel time. The knowledge, attitudes and practices reported may also differ considerably from those of the general population. Additional studies are needed to clarify these points. These studies should not be restricted to people who actively seek treatment preferably employing more in-depth interviewing techniques.

5.5. Tables

Table 5.1. Name of the disease, total respondents and respondents by sex and education level. The percentages for each group are given in parentheses and rounded to nearest integer. The four combinations of multiple names are unique (see text), three of them included *Aleppo sore* which occurred in combinations only.

	Total	Sex		Education, years	
Name of the disease		M	F	up to 6	7+
one-year sore	55 (79%)	17 (77%)	38 (79%)	37 (90%)	16 (62%)
Leishmania	3 (4%)	1 (5%)	2 (4%)	0	2 (8%)*
common pimple	6 (9%)	2 (9%)	4 (8%)	4 (10%)	2 (8%)
multiple names	4 (6%)	1 (5%)	3 (6%)	0	4 (15%)

* Missing educational level for one respondent.

Table 5.2. Causes/sources of leishmaniasis. Respondents might name more than one cause/source.

Source/cause	**n**
Insect	55
sandflies	*31*
mosquito	*13*
flies	*13*
unspecified	*5*
Man	12
Microbes	12
Leishmania	*1*
Dirt	8
Water	7
Other	4

Table 5.3. Causes/sources of leishmaniasis, ordered by rank of mentioning by the respondents divided by sex and education. Data on the level of education were available for 67 respondents. The respondents could give more than one answer in this category. For this reason the sums of numbers of answers may total to more than the number of respondents.

	Sex		Education	
Rank (number of responses)	*M (n=22)*	*F (n=48)*	*up to 6 years (n=41)*	*7 years or more (n=26)*
1	sandfly (8)	sandfly (25)	sandfly (22)	sandfly (10)
2	mosquito (5)	mosquito (10)	flies (10)	mosquito (8)
3	water (4)	man (10)	man (7)	microbes (6)
4	microbes (4)	flies (10)	microbes (6)	man (4)

Table 5.4. Knowledge: the most commonly mentioned cutaneous leishmaniasis prevention options, ordered by rank of mentioning, for the complete sample, males and in persons with educational level up to six years. The responses of females and those with more than six years in education had exactly the same order as in the complete sample.

Rank*	*Complete sample*	*Males*	*up to 6 years education*
1	bednets (17)	personal hygiene (3)	insecticides (10)
2	insecticides (14)	window mesh (3)	bednets (9)
3	personal hygiene (9)	sterilise water (2)	personal hygiene (6)
4	waste evacuation (5)**	waste evacuation (2)	waste evacuation (3)

* The ranks of responses of females and persons with more than six years of education was similar to that in the whole sample.

** There were two responses for waste evacuation from persons with more than six years of education. This group also gave two responses for each of *window mesh* and *wash fruits*.

Table 5.5. Service quality assessment by the respondents for patients who were followed up until cure was documented and those whose cure was not documented. Number of responses for every mark is given: 1, very bad; 2, bad; 3, acceptable; 4, good; 5, very good.

Service quality assessment	**1**	**2**	**3**	**4**	5	**Total**
Followed up until cure	0	0	0	11	4	**15**
Cure not recorded	3	2	3	2	0	**10**

Chapter 6. Discussion

In the current study several aspects of cutaneous leishmaniasis (CL) in Aleppo, Syria were studied. The clinical and epidemiological characteristics of the patients who attended primary health care (PHC) centres in Aleppo during 2005 were retrospectively analysed using the available routinely collected data and the course of treatment was explored in a subset of patients from PHC centre treatment registers and patient cards.

The clinical course of the disease was studied. Biological material was obtained from the lesions of patients before and in the course of treatment from which *Leishmania* parasites isolated. Isolates were tested in amastigote–macrophage system for sensitivity to antimonial drugs, amphotericin B, miltefosine and paromomycin.

Interviews were held with patients or accompanying adults to explore their knowledge about the disease, treatment-seeking behaviour and attitudes and practices regarding prevention, diagnosis and treatment.

A typical patient with CL is a child or adolescent who presents with one non-ulcerated lesion with reported duration of two months. Typically the patient undergoes one course of six injections and not infrequently stops treatment before the health centre staff tell them they are cured.

Lesion duration of two months was associated with the highest yield of smear positivity. On the other hand, patients with lesion duration of two months or longer received fewer injections and had shorter treatment duration. This suggests that active immunity to the parasite develops around two months from lesion appearance and contributes to faster cure. A similar notice was made in a study of the clinical course of cutaneous leishmaniasis in Peru, caused mostly by species of the *Viannia* subgenus (*L. peruviana, L. braziliensis* and *L. guayanensis*) [Llanos-Cuentas *et al.*, 2008], where disease duration of less than five weeks was associated with higher risk of treatment failure. Given this,

earlier diagnosis and start of treatment may worsen the prognosis for individual patients. On the other hand, if the risk for the sandfly acquiring infection from a lesion is correlated with smear positivity, which in turn is probably correlated with local parasite load, delaying the start of treatment may increase the risk of infection for the sandflies and, consequently, of disease transmission between humans.

More research is needed to better understand the human immune response to CL, possible non-human reservoirs of infection, the lifestyle of the known vector, *Ph. sergenti,* and possible other vectors of cutaneous leishmaniasis in Aleppo.

Given that only about half of our patients could be followed up until cure despite attempts to contact them, it can be concluded that a significant proportion of patients are not treated until cure, thus representing a potential reservoir for parasites that are primary-resistant in the clinical sense. This is probably related to the indolent nature of the disease itself and, in contrast, to painful and lengthy treatment. Since we avoided to select the PHC centres for this study where a proportion of population are likely to leave for harvest seasons, and tried to contact the patients who missed their appointments, it can be expected that patient compliance in Aleppo as a whole is even lower.

The leishmaniasis control strategy based on early detection, treatment until cure and vector control seems to fail in reducing the incidence of CL in Aleppo [Jalouk *et al.*, 2007], and the weak link is the treatment until cure. The other two components are probably also suboptimal. The results of treatment course analysis and *in vitro* assays in this study suggest that the real effect of intralesional pentavalent antimonial treatment of CL in Aleppo may be due mostly to physical local destruction of the tissues.

Jalouk *et al.* (2007) reported the results of insecticide-treated nets (ITNs) study in Aleppo governorate which were encouraging and suggested incorporating ITNs in the leishmaniasis control programme. But this

intervention requires substantial funds, and providing the nets on commercial basis by the MoH is hampered by imperfections in the legislation, according to which the PHC centres do not sell materials or medicines. In addition to ITNs, a relatively simple and inexpensive option deserves to be explored, covering any skin lesions that persist for longer than two weeks, at least during nights, to prevent the sandflies reaching them [Dr. Lama Jalouk, personal communication].

Less painful, shorter and more efficient treatment and/or prevention (e.g. vaccine) options are urgently needed but none are available to date.

Any treatment to be proposed should also be free of serious potential long-term side effects because most patients are young (75% are 25 years old or younger) and the disease is not life-threatening. This makes, for example, oral miltefosine not suitable for its treatment because of its teratogenic potential. However, for the rare cases of chronic mutilating disseminated disease that does not respond to systemic antimonials, drugs with potential efficacy may worth trying. This may include miltefosine or amphotericin B. A recent study reported the efficacy of a novel oral formulation or amphotericin B in mice [Wasan *et al.*, 2009]. This formulation had low toxicity in earlier studies, and if its efficacy and safety is confirmed in humans it can be an acceptable alternative to the current intralesional treatment. However, the cost may prove prohibitive. A novel topical aminoglycoside formulation was tested against *L. major* infection in Tunisia with significant efficacy [Ben Salah *et al.*, 2009], although about two-thirds of placebo patients also achieved cure. Paromomycin in Aleppo is not likely to be successful, given our *in vitro* results for paromomycin.

Our *in vitro* sensitivity data suggest that the parasites isolated from Aleppo are remarkably insensitive to pentavalent antimonials (mean and median EC_{50} values greater than 200 μg Sb^V/ml). All the isolates that were tested for sensitivity to paromomycin had EC_{50} values higher than the maximum test

concentration of 50 μM but all the isolates were highly sensitive to amphotericin B (mean and median EC_{50} values of about 67 nM). No meaningful association was found between *in vitro* parasite sensitivity and clinical outcome. This disagrees with a study of *L. tropica* in Iran [Hadighi *et al.*, 2006] but is in agreement with another study from Peru [Yardley *et al.*, 2006] that found no correlation between *in vitro* parasite sensitivity and outcome of infection with Latin American strains.

More than half of our isolates had EC_{50} values for Sb^V higher than the maximum assay concentration. This might be partly attributed to promastigotes that obscured the vision and probably affected the results. Ways to minimise this effect, either by modifying the assay protocol to get rid of promastigotes or using automated counting that is able to tell amastigotes from promastigotes, are needed. The promastigotes in our assay might come from the proportion of non-metacyclic dividing parasites that continued to multiply while the assay was being conducted. Metacyclics can be selected to improve infectivity by using lectin peanut agglutinin [da Silva and Sacks, 1987] or by pre-conditioning of the promastigotes by culturing in low pH [Inocêncio da Luz *et al.*, 2009]. Both these studies were performed on parasites other than *L. tropica*.

Epidemiological cycles and clinical characteristics of CL in the adjacent governorates of Idlib, Lattakia and Tartous, where the disease is believed to have emerged recently, should be investigated, with typing of the causative organisms and potential vectors.

The social study showed that a significant proportion of CL patients held views about disease causes, transmission and prevention that contradict contemporary scientific data. This shows there is room for improvement in health education delivery in Aleppo.

The disease was well recognised by our respondents but this may not be representative of the general population because the study was health-facility, rather than community based. This fact significantly limits the generalisability

of our results, and more systematic, community-based, studies are needed. Most respondents rated health services as good, and this response was associated with increased probability of treatment compliance.

Appendices

Appendix 1. Reporting health centres

	Dataset		
Health centre	**Main**	**Treatment**	**Patient Cards**
Abtin*	*NA*	49	*NA*
Abzmu*	*NA*	37	*NA*
Anadan*	*NA*	46	*NA*
Ashrafiyyeh	1281	*NA*	90
Atareb*	1169	240	2
Ayadat Shamileh	207	*NA*	19
Bab*	635	324	73
Batbu*	*NA*	39	*NA*
Efrin*	96	46	*NA*
Halab al-Jadidah	237	168	23
Hamadaniyyeh	1170	*NA*	22
Haydariyyeh	55	*NA*	*NA*
Hritan*	681	152	9
Jamal Abd al-Naser	337	*NA*	*NA*
Khalid ibn Walid	1435	*NA*	37
Khalidiyyeh	*NA*	478	*NA*
LCC	3051	391	62
Nayrab	314	90	28
Nizar al-Homsi	1619	*NA*	37
Salah al-Din	296	85	41
Sheikh Said	652	*NA*	22
Shuhadaa	148	*NA*	*NA*
Yousef al-Azmeh	545	*NA*	3
al-Jalaa	440	*NA*	30
Total	**14368**	**2145**	**498**

* Rural health centre. *NA*, no cases available in corresponding dataset.

Appendix 2. The health centres that report leishmaniasis cases in Aleppo

No.	Centre type	Centre name	borough/village (coverage)	population, 2003	ACL 2003	Incid. rate, ‰
1.	*Main centre*	*LCC*	*Aleppo governorate*		*2,103*	
2.	**Centre names in the city**	Nizar Homsi	Sheikh Maqsud	108,601	649	0.6
3.		Khaled ibn Walid	Khalidiyyeh	21,685	882	4.1
4.		Ashrafiyyeh	Ashrafiyyeh	84,174	501	0.6
5.		Jamal abdul-Naser	Hulluk	18,151	285	1.6
6.		Haydariyyeh	Masaken Hananu	118,138	323	0.3
7.		Yousef al-Azmeh	Tariq al-Bab	67,411	406	0.6
8.		Jalaa	Qadi Askar	25,243	579	2.3
9.		Mukhayyam Nayrab	Mukhayyam Nayrab	14,254	265	1.9
10.		Sheikh Said	Sheikh Said	8,114	492	6.1
11.		Shuhada	Sukkari	43,473	236	0.5
12.		Salah al-Din	Salah al-Din	36,364	324	0.9
13.		Ayadat Shamileh	Hamadaniyyeh	38,651	703	1.8
14.		Halab al-Jadidah	Halab al-Jadidah	21,963	197	0.9
15.	**Health posts,* city**	Mukhayyam Handarat	Mukhayyam Handarat	4,628	662	14
16.		Bani Zayd	Bani Zayd	7,462	720	9.6
	City subtotal			**618,312**	**9,327**	1.5
17.	**Centre names in the countryside**	Efrin	Efrin	38,977	34	0.08
18.		Hritan	Hritan	15,901	775	4.9
19.		Bab	Bab	61,629	135	0.2
20.		Hader	Hader	8,728	(b)	
21.		Atareb	Atareb	18,260	638	3.5
22.		Abzmu	Abzmu	5,369	7	0.1
23.		Kafr Daêl	Kafr Daêl	3,416	(b)	
24.		Hayyan	Hayyan	8,225	(b)	
25.		Anadan	Anadan	11,807	(b)	
26.		Batbu	Batbu	4,031	(b)	
27.		Ais	Ais	4,519	(b)	
28.		Abtin	Abtin	1,684	(b)	
29.		Blas	Blas	1,476	(b)	
30.		Aynjara	Aynjara	4,069	(b)	
31.	**Health posts,* countryside**	Kafr Karmin	Kafr Karmin	1,165	(b)	
32.		Tqad	Tqad	2,681	(b)	
33.		Dhahabiyyeh	Dhahabiyyeh	891	(b)	
34.		Kafr Amma	Kafr Amma	1,165	(b)	
35.		Shqaydleh	Shqaydleh	660	(b)	
	Countryside subtotal			**194,653**	**2,025**	1.0
	Total				**11,352**	

14 health centres in city
14 health centres in countryside
7 health posts in city and countryside

* Health posts work one day a week when they are visited by a qualified team from Leishmaniasis Control Centre or from a nearby PHC centre for diagnosis and treatment.

(b) Newly opened centres without lab diagnostic facilities; patients are sent to lab-equipped centres for confirmations and then treated in these new centres. No records for new patients are instituted.

Appendix 3. Consent form (original)

مقاومة الليشمانيا المدارية لمركبات الأنتموان في حلب

استمارة موافقة المريض

الباحث المسؤول: د. نزار أبازيد (هـ: XXX XX XX XX)

تصريح المريض:

- لقد قرأتُ صفحة المعلومات الخاصة بهذا البحث، أو فهمتُ الشرح الشفهي، وأنا أدرك ما هو المطلوب مني وماذا يحدث إذا شاركت في هذا البحث؛
- قام د. نزار أبازيد بالإجابة عن أسئلتي المتعلقة بهذا البحث؛
- أنا أعرف أنني أستطيع الانسحاب من الدراسة متى شئتُ دون أن أضطر إلى ذكر أسباب ذلك، وأن ذلك لن يؤثر بأي شكل على الرعاية والتدبير المعتاد؛
- لقد وافقتُ على المشاركة في هذا البحث.

وعدا المذكور أعلاه، فإنني:

- أوافق على أن يستخدم فريقُ الباحثين المعلومات حول عنواني ورقم هاتفي للاتصال بي من أجل المتابعة عند الضرورة. ❑ نعم ❑ لا
- أوافق على أن يتم تصوير الأفات، وأن يتم استخدام هذه الصور، **وبشرط أن هذه الصور لا تسمح بالتعرف على** شخصي، من أجل:
  - إضبارتي الطبية ❑ نعم ❑ لا
  - الأغراض البحثية ❑ نعم ❑ لا
  - الأغراض التعليمية ❑ نعم ❑ لا
  - النشر في المجلات الطبية أو الصحية ❑ نعم ❑ لا
  - النشر في مواقع الإنترنت الطبية أو الصحية ❑ نعم ❑ لا

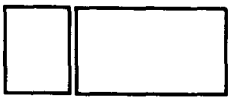
اسم المريض: ..... رقم التسجيل: ..... التوقيع: .....

أو (إذا كان المريض لا يعرف الكتابة):

الشاهد الأول: ..... الشاهد الثاني: .....

التاريخ: ...../...../2007 م

Appendix 4. Consent form (translation)



Tolerance of L. tropica to antimonials in Aleppo

Patient consent form

Researcher: Dr. Nizar Abazid (phone XXX XX XX XX)

Patient's declaration

- I read the information page, or understood oral explanation, and I understand what is required from me, and what happens if I decide to participate in this study;
- Dr. Nizar Abazid replied to my questions;
- I am aware I can withdraw from the study whenever I wish, and that I am not obliged to explain the reasons, and that my decision to withdraw will not affect in any way the usual health care and management I receive;
- I have agreed to participate in this study.

In addition to the above, I:

- agree that the study team use my contact details to contact me for follow up if necessary
  ❑ yes ❑ no
- agree to have my lesions photographed, and the images used, ***provided they do not allow to identify me***, for the following purposes:
  - my medical record ❑ yes ❑ no
  - research purposes ❑ yes ❑ no
  - teaching purposes ❑ yes ❑ no
  - publication in health and medical literature ❑ yes ❑ no
  - publication on medical or health internet sites ❑ yes ❑ no

Patient name: ..... patient ID: ..... signed: .....

or (if the patient is illiterate):

First witness ..... Second witness.....

Date: ...../...../200.....

Appendix 5. Study questionnaire

صحيفة بيانات إضافية

عن المصابين بداء الليشمانيات

اسم المركز الصحي	رمزه	رقم المريض	المخبري	المعالجة

بيانات المريض

رقم التعرف	اسم المريض:	كنيته:	اسم أبيه:	اسم أمه:
تاريخ الميلاد / /	العمر:	المستوى التعليمي للأطفال: المستوى التعليمي للأب: للأم:		
	الجنس:			
المهنة: للأطفال	مهنة الأب:		مهنة الأم:	
العنوان:	هـ		خليوي	

بيانات سريرية

تاريخ المراجعة: / /2007	تاريخ اللطاخة: / /2007	نتيجة اللطاخة:	اسم الفاحص:
عدد الآفات:	تاريخ ملاحظة أول افة: / 200	عمر الافة قبل المراجعة (أشهر):	

وصف الآفات عند المراجعة:

الآفة	نمطها	توضعها	قياساتها (مم)	ملاحظات
1			×	
2			×	
3			×	
4			×	
5			×	

الموافقة	الاجتماعية	الزرع	

ملاحظات

اسم منظم الاستمارة وتوقيعه:

سير العلاج

ر ت	الموعد	الفعلي	حجم الآفة					ملاحظات	الاسم	الحرز
			1	2	3	4	5			
	نجرعة الأولى	/ /								
2	/ /	/ /								
3	/ /	/ /								
4	/ /	/ /								
5	/ /	/ /								
6	/ /	/ /								
7	/ /	/ /								
8	/ /	/ /								
9	/ /	/ /								
10	/ /	/ /								
11	/ /	/ /								
12	/ /	/ /								
13	/ /	/ /								
14	/ /	/ /								
15	/ /	/ /								
16	/ /	/ /								
17	/ /	/ /								
18	/ /	/ /								
19	/ /	/ /								
20	/ /	/ /								
21	/ /	/ /								
22	/ /	/ /								
23	/ /	/ /								
24	/ /	/ /								

تاريخ شفاء الآفات: الأولى: / /2007، الثانية: / /2007، الثالثة: / /2007،

الرابعة: / /2007، الخامسة: / /2007، أو تاريخ الإحالة إلى مركز الليشمانيا: / /2007

زيارات المتابعة بعد إتمام العلاج

الزيارة	الموعد	الفعلي	حالة الافة					ملاحظات	الاسم
			1	2	3	4	5		
الأولى	/ /	/ /							
الثانية	/ /	/ /							
الثالثة	/ /	/ /							

Appendix 6. Study questionnaire (translation)

Data sheet
For leishmaniasis patients

Patient No.: Lab. Treat.		Health centre:

Patient data

mother	father	Surname	Name	ID
Education For children: father's mother's		Age Sex		DOB / /
mother	father	occupation		
mobile	tel.	address		

Clinical data

ex. by:	smear res:	smear date:	Referred:
lesion duration:		date first noted:	No. lesions

Lesion description (baseline)

Notes	Dimensions (mm)	Location	Type	Les.
	×			1
	×			2
	×			3
	×			4
	×			5

	Culture	Social	Consent

Notes

Course of treatment

score	name	notes	lesion size					actual	sched.	vis.
			5	4	3	2	1	/ /	first dose	
			×	×	×	✓	✓	/ /	/ /	2
			×	✓	✓	×	✓	/ /	/ /	3
			✓	✓	×	✓	✓	/ /	/ /	4
			×	×	✓	✓	✓	/ /	/ /	5
			✓	×	×	✓	×	/ /	/ /	6
			×	✓	✓	×	×	/ /	/ /	7
			×	×	×	✓	✓	/ /	/ /	8
			×	×	×	×	×	/ /	/ /	9
			×	×	×	×	×	/ /	/ /	10
			×	×	×	×	×	/ /	/ /	11
			×	×	×	×	×	/ /	/ /	12
			×	✓	×	×	×	/ /	/ /	13
			×	×	×	×	×	/ /	/ /	14
			×	×	×	×	×	/ /	/ /	15
			×	×	×	×	×	/ /	/ /	16
			×	×	×	×	×	/ /	/ /	17
			×	×	×	×	×	/ /	/ /	18
			×	×	×	×	×	/ /	/ /	19
			×	×	×	×	×	/ /	/ /	20
			×	×	×	×	×	/ /	/ /	21
			×	×	×	×	×	/ /	/ /	22
			×	×	×	×	×	/ /	/ /	23
			×	×	×	×	×	/ /	/ /	24

Lesions cured – first: / /200 ; second: / /200 ; third: / /200 ; fourth: / /200 fifth: / /200 ; or date of referral to LCC. / /200

Follow-up visits after completion of treatment

name	notes	lesion status					actual	sched.	visit
		5	4	3	2	1			
							/ /	/ /	first
							/ /	/ /	second
							/ /	/ /	third

Appendix 7. Medium M199

(for *Leishmania* spp. promastigotes

for 1 litre :

ddH_2O	1 litre
M199 powder (+ HEPES + l-glutamine)	Sigma M2520 1 litre units
$NaHCO_3$	2.2g
Adenosine	13.35mg
Hemin*	1ml - defrost
Pen/strep**	2ml - defrost

In a clean flask/beaker add the M199 powder to ~ 800ml ddH_2O. Mix well

Add 2.2g NaHCO3. Allow to mix well.

In a clean bijoux, dissolve the adenosine in 5ml 10mM NaOH. Add to medium and allow to mix well.

Add the pen/strep and hemin aliquots. Mix well.

Adjust to pH7.3 - 7.4 (use 5M HCl or 5M NaOH)

Filter sterilise into 2 x 500ml. Store at 4°C.

* 1M Hemin stocks are prepared: 25mg in 100ml 10mM NaOH. Freeze 1ml aliquots at -20°C.

**2ml aliquots of penicillin/streptomycin are stored at -20°C.

Appendix 8. Protocol of fingerprinting

I- DNA EXTRACTION (PHENOL/ CHLOROFORM)	I- DNA EXTRACTION (PHENOL/ CHLOROFORM)	I- DNA EXTRACTION (PHENOL/ CHLOROFORM)	I- DNA EXTRACTION (PHENOL/ CHLOROFORM)	I- DNA EXTRACTION (PHENOL/ CHLOROFORM)	I- DNA EXTRACTION (PHENOL/ CHLOROFORM)
Callibrated micropipettes, sterile barrier tips and tubes, sterile/molecular biology grade reagents in all steps. DNA extraction blanks used every 22 samples: 24 samples batch processed, blanks at positions 1 and 24	Callibrated micropipettes, sterile barrier tips and tubes, sterile/molecular biology grade reagents in all steps. DNA extraction blanks used every 22 samples: 24 samples batch processed, blanks at positions 1 and 25	Callibrated micropipettes, sterile barrier tips and tubes, sterile/molecular biology grade reagents in all steps. DNA extraction blanks used every 22 samples: 24 samples batch processed, blanks at positions 1 and 26	Callibrated micropipettes, sterile barrier tips and tubes, sterile/molecular biology grade reagents in all steps. DNA extraction blanks used every 22 samples: 24 samples batch processed, blanks at positions 1 and 27	Callibrated micropipettes, sterile barrier tips and tubes, sterile/molecular biology grade reagents in all steps. DNA extraction blanks used every 22 samples: 24 samples batch processed, blanks at positions 1 and 28	Callibrated micropipettes, sterile barrier tips and tubes, sterile/molecular biology grade reagents in all steps. DNA extraction blanks used every 22 samples: 24 samples batch processed, blanks at positions 1 and 29
1- *Leishmania* promastigotes pellet washing 1.1- Centrifugue original tubes: 3000 rpm 10 min 4 ºC 1.2- Transfer pellet and supernatant to 1.5 ml eppendorf trubes (screw cap) 1.3- Additional centrifugation (bench top microfugue): 6000 rpm 10 min RT 1.4- Discard supernatant (by pipeting) 1.5- Add 1 mL PBS 1X to remove ethanol trace 1.6- Centrifugation: 6000 rpm 10 min RT 1.7- Discard supernatant (by pipeting) 1.8- Store pellet at 4 ºC	**1- *Leishmania* promastigotes pellet washing** 1.1- Centrifugue original tubes: 3000 rpm 10 min 4 ºC 1.2- Transfer pellet and supernatant to 1.5 ml eppendorf trubes (screw cap) 1.3- Additional centrifugation (bench top microfugue): 6000 rpm 10 min RT 1.4- Discard supernatant (by pipeting) 1.5- Add 1 mL PBS 1X to remove ethanol trace 1.6- Centrifugation: 6000 rpm 10 min RT 1.7- Discard supernatant (by pipeting) 1.8- Store pellet at 4 ºC	**1- *Leishmania* promastigotes pellet washing** 1.1- Centrifugue original tubes: 3000 rpm 10 min 4 ºC 1.2- Transfer pellet and supernatant to 1.5 ml eppendorf trubes (screw cap) 1.3- Additional centrifugation (bench top microfugue): 6000 rpm 10 min RT 1.4- Discard supernatant (by pipeting) 1.5- Add 1 mL PBS 1X to remove ethanol trace 1.6- Centrifugation: 6000 rpm 10 min RT 1.7- Discard supernatant (by pipeting) 1.8- Store pellet at 4 ºC	**1- *Leishmania* promastigotes pellet washing** 1.1- Centrifugue original tubes: 3000 rpm 10 min 4 ºC 1.2- Transfer pellet and supernatant to 1.5 ml eppendorf trubes (screw cap) 1.3- Additional centrifugation (bench top microfugue): 6000 rpm 10 min RT 1.4- Discard supernatant (by pipeting) 1.5- Add 1 mL PBS 1X to remove ethanol trace 1.6- Centrifugation: 6000 rpm 10 min RT 1.7- Discard supernatant (by pipeting) 1.8- Store pellet at 4 ºC	**1- *Leishmania* promastigotes pellet washing** 1.1- Centrifugue original tubes: 3000 rpm 10 min 4 ºC 1.2- Transfer pellet and supernatant to 1.5 ml eppendorf trubes (screw cap) 1.3- Additional centrifugation (bench top microfugue): 6000 rpm 10 min RT 1.4- Discard supernatant (by pipeting) 1.5- Add 1 mL PBS 1X to remove ethanol trace 1.6- Centrifugation: 6000 rpm 10 min RT 1.7- Discard supernatant (by pipeting) 1.8- Store pellet at 4 ºC	**1- *Leishmania* promastigotes pellet washing** 1.1- Centrifugue original tubes: 3000 rpm 10 min 4 ºC 1.2- Transfer pellet and supernatant to 1.5 ml eppendorf trubes (screw cap) 1.3- Additional centrifugation (bench top microfugue): 6000 rpm 10 min RT 1.4- Discard supernatant (by pipeting) 1.5- Add 1 mL PBS 1X to remove ethanol trace 1.6- Centrifugation: 6000 rpm 10 min RT 1.7- Discard supernatant (by pipeting) 1.8- Store pellet at 4 ºC
2- Pellet Lysis 2.1- Add to each pellet: 400 µL NET10 buffer, 40 µL 10%SDS, 2 µL ProtK (20 mg/mL)	**2- Pellet Lysis** 2.1- Add to each pellet: 400 µL NET10 buffer, 40 µL 10%SDS, 2 µL ProtK (20 mg/mL)	**2- Pellet Lysis** 2.1- Add to each pellet: 400 µL NET10 buffer, 40 µL 10%SDS, 2 µL ProtK (20 mg/mL)	**2- Pellet Lysis** 2.1- Add to each pellet: 400 µL NET10 buffer, 40 µL 10%SDS, 2 µL ProtK (20 mg/mL)	**2- Pellet Lysis** 2.1- Add to each pellet: 400 µL NET10 buffer, 40 µL 10%SDS, 2 µL ProtK (20 mg/mL)	**2- Pellet Lysis** 2.1- Add to each pellet: 400 µL NET10 buffer, 40 µL 10%SDS, 2 µL ProtK (20 mg/mL)

I- DNA EXTRACTION (PHENOL/ CHLOROFORM)	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**
2.2- Mix by vortex	2.2- Mix by vortex	2.2- Mix by vortex	2.2- Mix by vortex	2.2- Mix by vortex	2.2- Mix by vortex
2.3- O/N incubation at 56 °C, shaking	2.3- O/N incubation at 56 °C, shaking	2.3- O/N incubation at 56 °C, shaking	2.3- O/N incubation at 56 °C, shaking	2.3- O/N incubation at 56 °C, shaking	2.3- O/N incubation at 56 °C, shaking
3- DNA purification	**3- DNA purification**	**3- DNA purification**	**3- DNA purification**	**3- DNA purification**	**3- DNA purification**
3.1- Briefly spin the tubes	3.1- Briefly spin the tubes	3.1- Briefly spin the tubes	3.1- Briefly spin the tubes	3.1- Briefly spin the tubes	3.1- Briefly spin the tubes
3.2- Add of 500 µL Phenol/ Chloroform/ Isoamylalcohol (25:24:1)	3.2- Add of 500 µL Phenol/ Chloroform/ Isoamylalcohol (25:24:1)	3.2- Add of 500 µL Phenol/ Chloroform/ Isoamylalcohol (25:24:1)	3.2- Add of 500 µL Phenol/ Chloroform/ Isoamylalcohol (25:24:1)	3.2- Add of 500 µL Phenol/ Chloroform/ Isoamylalcohol (25:24:1)	3.2- Add of 500 µL Phenol/ Chloroform/ Isoamylalcohol (25:24:1)
3.3- Mix by inversion 10 times	3.3- Mix by inversion 10 times	3.3- Mix by inversion 10 times	3.3- Mix by inversion 10 times	3.3- Mix by inversion 10 times	3.3- Mix by inversion 10 times
3.4- Centrifugue: 13000 rpm 6 min	3.4- Centrifugue: 13000 rpm 6 min	3.4- Centrifugue: 13000 rpm 6 min	3.4- Centrifugue: 13000 rpm 6 min	3.4- Centrifugue: 13000 rpm 6 min	3.4- Centrifugue: 13000 rpm 6 min
3.5- Transfer aqueous phase to 1.5 mL tubes containing 500 µL Chloroform/ Isoamylalcohol (24:1)	3.5- Transfer aqueous phase to 1.5 mL tubes containing 500 µL Chloroform/ Isoamylalcohol (24:1)	3.5- Transfer aqueous phase to 1.5 mL tubes containing 500 µL Chloroform/ Isoamylalcohol (24:1)	3.5- Transfer aqueous phase to 1.5 mL tubes containing 500 µL Chloroform/ Isoamylalcohol (24:1)	3.5- Transfer aqueous phase to 1.5 mL tubes containing 500 µL Chloroform/ Isoamylalcohol (24:1)	3.5- Transfer aqueous phase to 1.5 mL tubes containing 500 µL Chloroform/ Isoamylalcohol (24:1)
3.6- Discard the tubes containing the dirty-phenol phase	3.6- Discard the tubes containing the dirty-phenol phase	3.6- Discard the tubes containing the dirty-phenol phase	3.6- Discard the tubes containing the dirty-phenol phase	3.6- Discard the tubes containing the dirty-phenol phase	3.6- Discard the tubes containing the dirty-phenol phase
3.7- Centrifugue the tubes containing CI/IA-Aqueous phase: 13000 rpm 6 min	3.7- Centrifugue the tubes containing CI/IA-Aqueous phase: 13000 rpm 6 min	3.7- Centrifugue the tubes containing CI/IA-Aqueous phase: 13000 rpm 6 min	3.7- Centrifugue the tubes containing CI/IA-Aqueous phase: 13000 rpm 6 min	3.7- Centrifugue the tubes containing CI/IA-Aqueous phase: 13000 rpm 6 min	3.7- Centrifugue the tubes containing CI/IA-Aqueous phase: 13000 rpm 6 min
3.8- Transfer aqueous phase to 1.5 mL tubes containing:	3.8- Transfer aqueous phase to 1.5 mL tubes containing:	3.8- Transfer aqueous phase to 1.5 mL tubes containing:	3.8- Transfer aqueous phase to 1.5 mL tubes containing:	3.8- Transfer aqueous phase to 1.5 mL tubes containing:	3.8- Transfer aqueous phase to 1.5 mL tubes containing:
3.9- Mix by inversion 10 times	3.9- Mix by inversion 10 times	3.9- Mix by inversion 10 times	3.9- Mix by inversion 10 times	3.9- Mix by inversion 10 times	3.9- Mix by inversion 10 times
3.10- Keep the tubes at -70 °C for 20 min (or -20 °C O/N)	3.10- Keep the tubes at -70 °C for 20 min (or -20 °C O/N)	3.10- Keep the tubes at -70 °C for 20 min (or -20 °C O/N)	3.10- Keep the tubes at -70 °C for 20 min (or -20 °C O/N)	3.10- Keep the tubes at -70 °C for 20 min (or -20 °C O/N)	3.10- Keep the tubes at -70 °C for 20 min (or -20 °C O/N)
3.11- Centrifugue: 13000 rpm 6 min	3.11- Centrifugue: 13000 rpm 6 min	3.11- Centrifugue: 13000 rpm 6 min	3.11- Centrifugue: 13000 rpm 6 min	3.11- Centrifugue: 13000 rpm 6 min	3.11- Centrifugue: 13000 rpm 6 min
3.12- Discard supernatant	3.12- Discard supernatant	3.12- Discard supernatant	3.12- Discard supernatant	3.12- Discard supernatant	3.12- Discard supernatant
3.13- Add 1 mL 70% EtOH	3.13- Add 1 mL 70% EtOH	3.13- Add 1 mL 70% EtOH	3.13- Add 1 mL 70% EtOH	3.13- Add 1 mL 70% EtOH	3.13- Add 1 mL 70% EtOH
3.14- Mix by inversion 10 times	3.14- Mix by inversion 10 times	3.14- Mix by inversion 10 times	3.14- Mix by inversion 10 times	3.14- Mix by inversion 10 times	3.14- Mix by inversion 10 times
3.15- Centrifugue: 13000 rpm 6 min	3.15- Centrifugue: 13000 rpm 6 min	3.15- Centrifugue: 13000 rpm 6 min	3.15- Centrifugue: 13000 rpm 6 min	3.15- Centrifugue: 13000 rpm 6 min	3.15- Centrifugue: 13000 rpm 6 min

I- DNA EXTRACTION (PHENOL/ CHLOROFORM)	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**	**I- DNA EXTRACTION (PHENOL/ CHLOROFORM)**
3.16- Discard supernatant 3.17- Let the tubes to air dry RT	3.16- Discard supernatant 3.17- Let the tubes to air dry RT	3.16- Discard supernatant 3.17- Let the tubes to air dry RT	3.16- Discard supernatant 3.17- Let the tubes to air dry RT	3.16- Discard supernatant 3.17- Let the tubes to air dry RT	3.16- Discard supernatant 3.17- Let the tubes to air dry RT
4- DNA elution 4.1- Add 50 μL sterile distilled water to each tube 4.2- Store at 4 $^{\circ}C$ for 24h before use	**4- DNA elution** 4.1- Add 50 μL sterile distilled water to each tube 4.2- Store at 4 $^{\circ}C$ for 24h before use	**4- DNA elution** 4.1- Add 50 μL sterile distilled water to each tube 4.2- Store at 4 $^{\circ}C$ for 24h before use	**4- DNA elution** 4.1- Add 50 μL sterile distilled water to each tube 4.2- Store at 4 $^{\circ}C$ for 24h before use	**4- DNA elution** 4.1- Add 50 μL sterile distilled water to each tube 4.2- Store at 4 $^{\circ}C$ for 24h before use	**4- DNA elution** 4.1- Add 50 μL sterile distilled water to each tube 4.2- Store at 4 $^{\circ}C$ for 24h before use

II- REAGENTS USED FOR DNA EXTRACTION	**II- REAGENTS USED FOR DNA EXTRACTION**	**II- REAGENTS USED FOR DNA EXTRACTION**	**II- REAGENTS USED FOR DNA EXTRACTION**	**II- REAGENTS USED FOR DNA EXTRACTION**	**II- REAGENTS USED FOR DNA EXTRACTION**
1- NET 10 Buffer 10mM NaCl, 10mMEDTA, 10mM Tris HCl Autoclave and store at 4 $^{\circ}C$	**1- NET 10 Buffer** 10mM NaCl, 10mMEDTA, 10mM Tris HCl Autoclave and store at 4 $^{\circ}C$	**1- NET 10 Buffer** 10mM NaCl, 10mMEDTA, 10mM Tris HCl Autoclave and store at 4 $^{\circ}C$	**1- NET 10 Buffer** 10mM NaCl, 10mMEDTA, 10mM Tris HCl Autoclave and store at 4 $^{\circ}C$	**1- NET 10 Buffer** 10mM NaCl, 10mMEDTA, 10mM Tris HCl Autoclave and store at 4 $^{\circ}C$	**1- NET 10 Buffer** 10mM NaCl, 10mMEDTA, 10mM Tris HCl Autoclave and store at 4 $^{\circ}C$
2- 10%SDS Dilute SDS on sterile distilled water and store at RT	**2- 10%SDS** Dilute SDS on sterile distilled water and store at RT	**2- 10%SDS** Dilute SDS on sterile distilled water and store at RT	**2- 10%SDS** Dilute SDS on sterile distilled water and store at RT	**2- 10%SDS** Dilute SDS on sterile distilled water and store at RT	**2- 10%SDS** Dilute SDS on sterile distilled water and store at RT
3- PHENOL / CHLOROFORM / ISOAMYL ALCOHOL (25:24:1) Mix 25 parts of Phenol with 25 parts of Chloroform:Isoamyl alcohol (24:1) previously prepared Let two phases to appear, wrap with aluminum paper and store at 4 $^{\circ}C$	**3- PHENOL / CHLOROFORM / ISOAMYL ALCOHOL (25:24:1)** Mix 25 parts of Phenol with 25 parts of Chloroform:Isoamyl alcohol (24:1) previously prepared Let two phases to appear, wrap with aluminum paper and store at 4 $^{\circ}C$	**3- PHENOL / CHLOROFORM / ISOAMYL ALCOHOL (25:24:1)** Mix 25 parts of Phenol with 25 parts of Chloroform:Isoamyl alcohol (24:1) previously prepared Let two phases to appear, wrap with aluminum paper and store at 4 $^{\circ}C$	**3- PHENOL / CHLOROFORM / ISOAMYL ALCOHOL (25:24:1)** Mix 25 parts of Phenol with 25 parts of Chloroform:Isoamyl alcohol (24:1) previously prepared Let two phases to appear, wrap with aluminum paper and store at 4 $^{\circ}C$	**3- PHENOL / CHLOROFORM / ISOAMYL ALCOHOL (25:24:1)** Mix 25 parts of Phenol with 25 parts of Chloroform:Isoamyl alcohol (24:1) previously prepared Let two phases to appear, wrap with aluminum paper and store at 4 $^{\circ}C$	**3- PHENOL / CHLOROFORM / ISOAMYL ALCOHOL (25:24:1)** Mix 25 parts of Phenol with 25 parts of Chloroform:Isoamyl alcohol (24:1) previously prepared Let two phases to appear, wrap with aluminum paper and store at 4 $^{\circ}C$
4- CHLOROFORM /ISOAMYL ALCOHOL (24:1) Mix 24 parts of Chloroform with 1 part of Isoamyl alcohol	**4- CHLOROFORM /ISOAMYL ALCOHOL (24:1)** Mix 24 parts of Chloroform with 1 part of Isoamyl alcohol	**4- CHLOROFORM /ISOAMYL ALCOHOL (24:1)** Mix 24 parts of Chloroform with 1 part of Isoamyl alcohol	**4- CHLOROFORM /ISOAMYL ALCOHOL (24:1)** Mix 24 parts of Chloroform with 1 part of Isoamyl alcohol	**4- CHLOROFORM /ISOAMYL ALCOHOL (24:1)** Mix 24 parts of Chloroform with 1 part of Isoamyl alcohol	**4- CHLOROFORM /ISOAMYL ALCOHOL (24:1)** Mix 24 parts of Chloroform with 1 part of Isoamyl alcohol

II- REAGENTS USED FOR DNA EXTRACTION	**II- REAGENTS USED FOR DNA EXTRACTION**	**II- REAGENTS USED FOR DNA EXTRACTION**	**II- REAGENTS USED FOR DNA EXTRACTION**	**II- REAGENTS USED FOR DNA EXTRACTION**	**II- REAGENTS USED FOR DNA EXTRACTION**
Wrap with aluminum paper and store at 4 °C **5- ETHANOL ABSOLUTE & ETHANOL 70%** Store at -20 °C **6- 3M Na-Acetate pH 6.0** pH adjusted with Acetic Acid Autoclave and store at RT	Wrap with aluminum paper and store at 4 °C **5- ETHANOL ABSOLUTE & ETHANOL 70%** Store at -20 °C **6- 3M Na-Acetate pH 6.1** pH adjusted with Acetic Acid Autoclave and store at RT	Wrap with aluminum paper and store at 4 °C **5- ETHANOL ABSOLUTE & ETHANOL 70%** Store at -20 °C **6- 3M Na-Acetate pH 6.2** pH adjusted with Acetic Acid Autoclave and store at RT	Wrap with aluminum paper and store at 4 °C **5- ETHANOL ABSOLUTE & ETHANOL 70%** Store at -20 °C **6- 3M Na-Acetate pH 6.3** pH adjusted with Acetic Acid Autoclave and store at RT	Wrap with aluminum paper and store at 4 °C **5- ETHANOL ABSOLUTE & ETHANOL 70%** Store at -20 °C **6- 3M Na-Acetate pH 6.4** pH adjusted with Acetic Acid Autoclave and store at RT	Wrap with aluminum paper and store at 4 °C **5- ETHANOL ABSOLUTE & ETHANOL 70%** Store at -20 °C **6- 3M Na-Acetate pH 6.5** pH adjusted with Acetic Acid Autoclave and store at RT
III- DNA QUANTIFICATION	**III- DNA QUANTIFICATION**	**III- DNA QUANTIFICATION**	**III- DNA QUANTIFICATION**	**III- DNA QUANTIFICATION**	**III- DNA QUANTIFICATION**
System ND-1000 Spectrophotometer (NanoDrop®) Volume of sample assayed 2 μL, in duplicate. Records of cc (ng/μL) and A260 / A280 ratio were taken	System ND-1000 Spectrophotometer (NanoDrop®) Volume of sample assayed 2 μL, in duplicate. Records of cc (ng/μL) and A260 / A280 ratio were taken	System ND-1000 Spectrophotometer (NanoDrop®) Volume of sample assayed 2 μL, in duplicate. Records of cc (ng/μL) and A260 / A280 ratio were taken	System ND-1000 Spectrophotometer (NanoDrop®) Volume of sample assayed 2 μL, in duplicate. Records of cc (ng/μL) and A260 / A280 ratio were taken	System ND-1000 Spectrophotometer (NanoDrop®) Volume of sample assayed 2 μL, in duplicate. Records of cc (ng/μL) and A260 / A280 ratio were taken	System ND-1000 Spectrophotometer (NanoDrop®) Volume of sample assayed 2 μL, in duplicate. Records of cc (ng/μL) and A260 / A280 ratio were taken
IV- PCR METHOD	**IV- PCR METHOD**	**IV- PCR METHOD**	**IV- PCR METHOD**	**IV- PCR METHOD**	**IV- PCR METHOD**
PCR method: based on Aransay *et al Appl Enviroment Microbiol* 2000;66:1933-38 **Reference strain:** *Leishmania tropica* K27 **PCR MIX** **Reagents**: Biotools B&M LABS (www.biotools.eu) > Buffer 10X (20 mM Mg Cl2) > dNTP mix (10 mM each)	**PCR method:** based on Aransay *et al Appl Enviroment Microbiol* 2000;66:1933-39 **Reference strain:** *Leishmania tropica* K28 **PCR MIX** **Reagents**: Biotools B&M LABS (www.biotools.eu) > Buffer 10X (20 mM Mg Cl2) > dNTP mix (10 mM each)	**PCR method:** based on Aransay *et al Appl Enviroment Microbiol* 2000;66:1933-40 **Reference strain:** *Leishmania tropica* K29 **PCR MIX** **Reagents**: Biotools B&M LABS (www.biotools.eu) > Buffer 10X (20 mM Mg Cl2) > dNTP mix (10 mM each)	**PCR method:** based on Aransay *et al Appl Enviroment Microbiol* 2000;66:1933-41 **Reference strain:** *Leishmania tropica* K30 **PCR MIX** **Reagents**: Biotools B&M LABS (www.biotools.eu) > Buffer 10X (20 mM Mg Cl2) > dNTP mix (10 mM each)	**PCR method:** based on Aransay *et al Appl Enviroment Microbiol* 2000;66:1933-42 **Reference strain:** *Leishmania tropica* K31 **PCR MIX** **Reagents**: Biotools B&M LABS (www.biotools.eu) > Buffer 10X (20 mM Mg Cl2) > dNTP mix (10 mM each)	**PCR method:** based on Aransay *et al Appl Enviroment Microbiol* 2000;66:1933-43 **Reference strain:** *Leishmania tropica* K32 **PCR MIX** **Reagents**: Biotools B&M LABS (www.biotools.eu) > Buffer 10X (20 mM Mg Cl2) > dNTP mix (10 mM each)

IV- PCR METHOD	IV- PCR METHOD	IV- PCR METHOD	IV- PCR METHOD	IV- PCR METHOD	IV- PCR METHOD
> primers LIN R4 and LIN 19 synthesized by SIGMA Genosys > Tth DNA pol (1U/ µL)	> primers LIN R4 and LIN 19 synthesized by SIGMA Genosys > Tth DNA pol (1U/ µL)	> primers LIN R4 and LIN 19 synthesized by SIGMA Genosys > Tth DNA pol (1U/ µL)	> primers LIN R4 and LIN 19 synthesized by SIGMA Genosys > Tth DNA pol (1U/ µL)	> primers LIN R4 and LIN 19 synthesized by SIGMA Genosys > Tth DNA pol (1U/ µL)	> primers LIN R4 and LIN 19 synthesized by SIGMA Genosys > Tth DNA pol (1U/ µL)
PCR MIX	PCR MIX	PCR MIX	PCR MIX	PCR MIX	PCR MIX
REAGENT	REAGENT	REAGENT	REAGENT	REAGENT	REAGENT
H2O	H2O	H2O	H2O	H2O	H2O
Buffer 10X	Buffer 10X	Buffer 10X	Buffer 10X	Buffer 10X	Buffer 10X
dNTP mix	dNTP mix	dNTP mix	dNTP mix	dNTP mix	dNTP mix
LINR4 (10 pmol/µL)	LINR4 (10 pmol/µL)	LINR4 (10 pmol/µL)	LINR4 (10 pmol/µL)	LINR4 (10 pmol/µL)	LINR4 (10 pmol/µL)
LIN19 (10 pmol/µL)	LIN19 (10 pmol/µL)	LIN19 (10 pmol/µL)	LIN19 (10 pmol/µL)	LIN19 (10 pmol/µL)	LIN19 (10 pmol/µL)
Tth Dna pol	Tth Dna pol	Tth Dna pol	Tth Dna pol	Tth Dna pol	Tth Dna pol
DNA template	DNA template	DNA template	DNA template	DNA template	DNA template

V- RFLP	V- RFLP	V- RFLP	V- RFLP	V- RFLP	V- RFLP
17 µL PCR product	18 µL PCR product	19 µL PCR product	20 µL PCR product	21 µL PCR product	22 µL PCR product
2 µL Buffer 10X	3 µL Buffer 10X	4 µL Buffer 10X	5 µL Buffer 10X	6 µL Buffer 10X	7 µL Buffer 10X
1 µL enzyme (HaeIII / RsaI)	2 µL enzyme (HaeIII / RsaI)	3 µL enzyme (HaeIII / RsaI)	4 µL enzyme (HaeIII / RsaI)	5 µL enzyme (HaeIII / RsaI)	6 µL enzyme (HaeIII / RsaI)
37°C O/N	37°C O/N	37°C O/N	37°C O/N	37°C O/N	37°C O/N
Electrophoresis 2% agarose	Electrophoresis 2% agarose	Electrophoresis 2% agarose	Electrophoresis 2% agarose	Electrophoresis 2% agarose	Electrophoresis 2% agarose

Appendix 9. Social study questionnaire

استمارة الدراسة الاجتماعية

رقم التعرف		العمر	الجنس	المجيب	عمره	الجنس	التاريخ: / / ٢٠٠
المعارف							
1-	اسم المرض						
2-	سببه						
3-	كيفية الإصابة						
4-	إمكانية الوقاية						
5-	مدة المرض						
6-	رجعة المرض						
طلب العلاج وإتاحته							
1-	من لاحظ الآفة						
2-	ماذا لاحظت أولا						
3-	ماذا فعلت أولا						
4-	النصائح						
5-	من نصح بالعلاج						
6-	المدة من الملاحظة						
7-	الإحالة إلى المركز						
8-	صاحب القرار						
9-	المرافقون						
التوقعات							
1-	طبيعة العلاج						
2-	مدة العلاج						
3-	علامات الشفاء						
المواقف							
1-	ضرورة الوقاية						
2-	ضرورة المعالجة						
3-	الأعراض المزعجة						
الممارسات							
1-	النوم خارج الغرفة						
2-	استعمال الكلل						
3-	استعمال المبيدات						

ملاحظات

المتابعة	المجيب:	التاريخ: / / 200

الانطباع عن جودة الرعاية

وقت الوصول

زمن الانتظار

الانطباع عن مواقف العاملين

المنقطعون عن العلاج (ألم، تقييم تطور الآفة، زمن الوصول والانتظار، **موافق العاملين**، إلخ)

Appendix 10. Social study questionnaire (translation)

Leishmaniasis in Aleppo, Syria
Social study questionnaire

PID	Sex	Age	Respondent	Age	Edu	Interviewed	

Knowledge

1. *What do you name this problem?*
2. *What is the cause of this problem?*
3. *How do people get the disease (is it contagious, can it be contracted by simple contact, transmitted by flies, mosquitoes, etc.)?*
4. *Can the disease be prevented? If yes, how? (avoiding affected persons, insecticides, sleeping indoors/outdoors, bed nets)*
5. *How long it takes to cure?*
6. *How often the does the disease affect the same person again?*

Treatment seeking and access

1. *Who noticed the problem first, was it you or someone else?*
2. *What did you first notice?*
3. *What did you first do?*
4. *What advice were you given?*
5. *Who advised the patient to seek treatment? Who participated in decision-taking?*
6. *How long did it take from disease recognition and seeking treatment?*
7. *Who referred the patient to this centre?*
8. *Did the patient himself make the decision to seek treatment/whose permission was required? Any other problems with access to the clinic?*
9. *Who accompanies the patient?*

Expectations

1. *What is the nature of treatment?*
2. *How long does the treatment take?*
3. *How do you know you are cured?*

Attitudes

1. *Should people try to prevent the disease? How? Whose responsibility is this (government/individuals)?*
2. *Is it necessary to treat the disease? If yes, why? What happens if lesions are left untreated? Why treating the disease is better/worse?*
3. *What are most distressing manifestations and consequences of the disease?*

Practices

a)	*do you sleep outdoors?*	
b)	*do you use bed nets?*	
c)	*do you spray insecticides?*	

Perceived quality of care

Travel time
Waiting time
Perceived staff attitude

Patients prematurely stopping their treatment

References

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