T.K. Jha
E. Noiri *Editors*

Kala Azar in South Asia

Current Status and Challenges Ahead

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ISBN 978-94-007-0276-9 e-ISBN 978-94-007-0277-6
DOI 10.1007/978-94-007-0277-6
Springer Dordrecht Heidelberg London New York

Preface

One of the most important challenges for third world countries is to educate and support their inhabitants to maintain an independent and comfortable lifestyle. Difficult enough when there is no health problem, but life-threatening infectious diseases are more prevalent in the tropical zone than in more temperate climates. Compared to other tropical infectious diseases, patients with Visceral Leishmaniasis (VL) won't visit the clinic until the disease developed for longer periods and therefore they are often in the advanced stages of the disease: the symptoms do not appear early and the disease process is subacute and does not greatly affect the physical wellbeing.

The diagnosis of VL used to be extremely difficult for physicians, requiring a bone marrow biopsy, but the recent improvements made the diagnosis much easier and faster. When treating patients, the adherence to medication is always an issue for medical practice, even the western countries but especially so in the developing world. To cope with this and other issues, therapeutic patterns have to be developed and evaluated specifically for the endemic areas.

During the next several years, the clinical approach to VL and to post kala-azar dermal Leishmaniasis in the Indian subcontinent and South-Asia will need to change. Standing at a turning point in VL medicine, we had the opportunity to organize the expert conference entitled "Consultative Meeting on Elimination of Kala-Azar from South Asia" in the late summer of 2009 in New Delhi, India under the support of Japanese Science Technology Agency. It is my great pleasure to share with you the latest knowledge in Visceral Leishmaniasis!

Tokyo, Japan — Eisei NOIRI, MD, PhD

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Part I
Introduction into Kala-Azar

Chapter 1
Geographical Distribution of Kala-Azar in South Asia

Moazzem Hossain and Kazi M. Jamil

Abstract Kala-azar or visceral leishmaniasis (VL) is a parasitic disease caused by the trypanosomatid parasite *Leishmania donovani* on the Indian subcontinent, where it is transmitted by the sandfly *Phlebotomus argentipes*. Leishmaniasis is found in about 88 countries, where 350 million people are believed to be at risk. About 500,000 cases of VL occur annually, mostly affecting countries in the tropics and subtropics. More than 90% of the world's cases of VL occur in India, Bangladesh, Nepal, Sudan, and Brazil, affecting largely the socially marginalized and the poorest communities. In South Asia, kala-azar occurs in India, Bangladesh, and Nepal, with a small focus reported from Bhutan. Nearly 200 million people are considered to be at risk of contracting kala–azar in this region. In May 2005, the health ministers of these three countries signed a memorandum of understanding in Geneva during the World Health Assembly, making a commitment to eliminate kala-azar from their respective countries by 2015. The target of the elimination program is to reduce the incidence of kala-azar to less than one case of kala-azar or post-kala-azar dermal leishmaniasis per 10,000 population at the district (in Nepal) or subdistrict/upazila level (in Bangladesh and India). As of 2008, kala-azar was endemic in 52 districts in India, 12 districts in Nepal, and 45 districts in Bangladesh.

Keywords Indian subcontinent · Visceral leishmania

Abbreviations

DDT	Dichlorodiphenyltrichloroethane
RTAG	Regional technical advisory group
SAG	Sodium antimony gluconate
VL	Visceral leishmaniasis
WHO	World Health Organization

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_1,

1.1 Global and Regional Situation

Kala-azar or visceral leishmaniasis (VL) is one of the complex of diseases called leishmaniasis. It is caused by the trypanosomatid parasite *Leishmania donovani.* On the Indian subcontinent, it is transmitted by the sandfly *Phlebotomus argentipes*. The disease presents with fever of long duration (more than 2 weeks), along with splenomegaly, anemia, and progressive weight loss. In endemic areas, children and young adults are its principal victims. The disease is usually fatal if the patient is left without treatment. Human immunodeficiency virus coinfection in kala-azar patients has emerged as a health problem in recent years [1] Fig. 1.1.

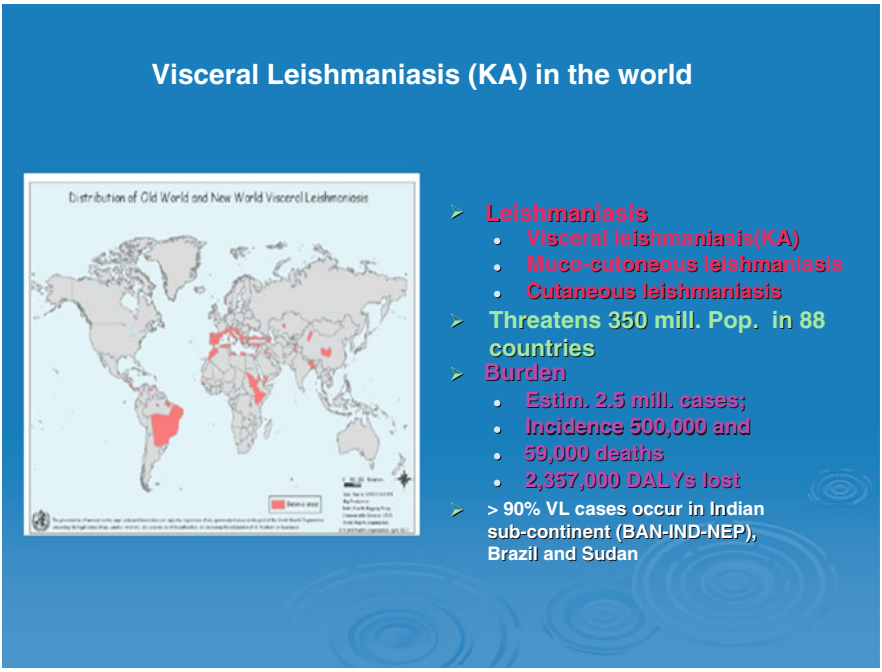

Fig. 1.1 Global burden of visceral leishmaniasis

Leishmaniasis is found in about 88 countries, with a combined population of approximately 350 million. About 500,000 cases of VL occur annually. Most of the affected countries are in the tropics and subtropics. The settings in which leishmaniasis is found range from rain forests in Central and South America to deserts in West Asia. More than 90% of the world's cases of VL are in India, Bangladesh, Nepal, Sudan, and Brazil, affecting largely the socially marginalized and the poorest communities.

In the South-East Asian Region, kala-azar occurs in India, Bangladesh, and Nepal. A small focus has also been reported from Bhutan. In the three countries

of the region, about 200 million people in 109 districts are "at risk". In India, 52 districts in the four states of Bihar, Jharkhand, West Bengal, and Uttar Pradesh (certain eastern districts) are currently endemic for the disease. In Nepal, 12 districts contiguous to the states of Bihar and Uttar Pradesh are endemic, while in Bangladesh, kala-azar has been reported in 45 districts. In December 2003, India launched a large-scale kala-azar elimination program with the objective of eliminating the disease by 2010. Bangladesh and Nepal are also committed to a kala-azar elimination program, with the target of achieving the elimination of the disease by 2015. There is a great deal of political commitment in the region to the elimination of kala-azar. In May 2005, these three countries signed a memorandum of understanding in Geneva during the World Health Assembly, pledging to engage in mutual cooperation to eliminate kala-azar from their respective countries. A regional strategic plan supporting elimination of the disease was prepared and endorsed by the Regional Technical Advisory Group (RTAG) of the South-East Asia Regional Office of the World Health Organization (WHO) and its partners. The second meeting of the RTAG held in Kathmandu in October 2006 recommended that WHO prepare guidelines and standard operating procedures to ensure that measures undertaken in the endemic countries are uniformly applied Fig. 1.2.

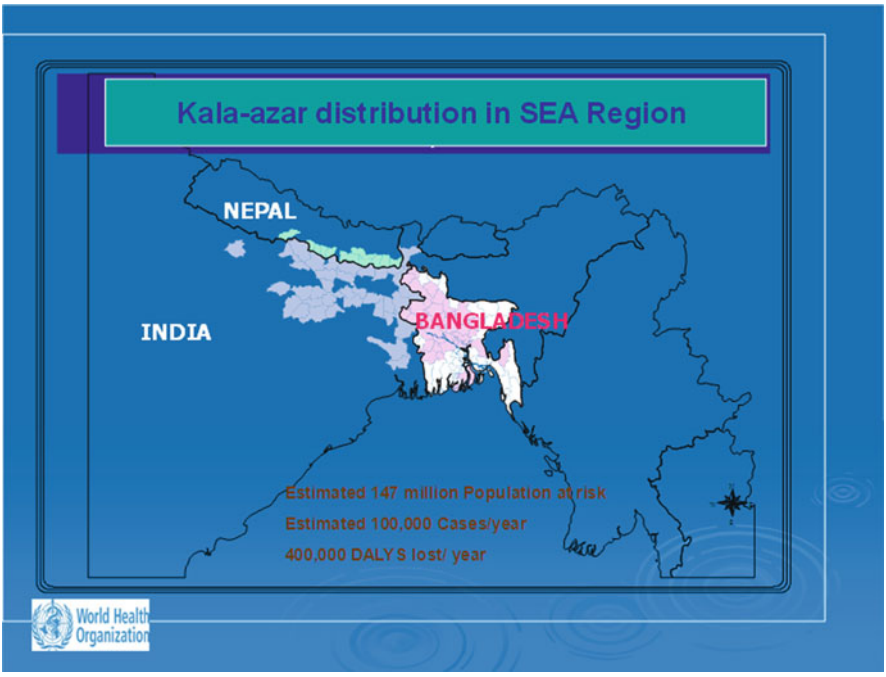

Fig. 1.2 Distribution of Kala-azar in South Asia

1.2 Kala-Azar in Bangladesh

Kala-azar is one of the major public health problems in Bangladesh, where the disease has been endemic for many decades. Blanket spraying of dichlorodiphenyltrichloroethane (DDT) during the WHO-supported malaria eradication program in the 1960s controlled kala-azar transmission, but in the late 1970s, kala-azar re-emerged sporadically. Between 1981 and 1985, only eight upazilas (subdistricts) reported cases of kala-azar—a figure that increased to 105 upazilas in 2004. Over the past few years, kala-azar has assumed epidemic proportions, with the number of reported cases increasing from 3,978 in 1993 to 8,505 in 2005. Surveillance is weak at present, and the current estimated total cases number about 45,000. Annually, approximately 10,000 cases are treated under the elimination program, but cases treated by private clinics and practitioners are not reported. It has been estimated that the true incidence of VL in Bangladesh is five times higher than the figure recorded by government surveillance [2] Fig. 1.3.

Under the current surveillance system, the Upazila Health Complexes, District Hospitals and other specialized hospitals report cases to the disease control unit of the Directorate General of Health Services. This, however, results in a gross under-reporting of cases, because private-sector clinics and hospitals are not included, nor are cases treated by private practitioners. At the time of this writing (January 2010), sodium antimony gluconate (SAG) administered by injection was the only drug being used for VL treatment under the kala-azar elimination program in Bangladesh, and was being administered to VL patients free of charge. Passive surveillance works only when drugs are available in hospitals so that the VL cases treated can be monitored.

Kala–azar cases are usually clustered in villages displaying environmental and other factors that encourage the growth and proliferation of vectors. There is no marked seasonal variation, but peaks in transmission are suggested by the pre- and post-monsoon rise in the number of cases. Areas in the Old Brahmaputra and Ganges basins show the highest prevalence of the disease.

Figures 1.4 and 1.5 show, by district, the incidence of kala-azar and the mortality rate of patients between 1999 and 2008. The highest mortality rate, recorded in the Mymensingh district, was 6.4% [3]. However, definite data on morbidity and mortality due to kala-azar are not available under the current reporting system, and a breakdown of the data according to age and gender has not been provided.

P. argentipes is the vector of kala-azar in Bangladesh. The vector habitat is restricted to domiciliary and peridomicillary areas. Favorable conditions for sandfly multiplication, include (a) a monthly mean temperature between 7.2°C and 37°C; (b) mean annual relative humidity of 70%, with 80% for at least 3 months; (c) annual rainfall of 1,250 mm or more; (d) altitude of <600 m; (e) alluvial soil; (f) high groundwater levels; and (g) abundant vegetation. These conditions are encountered in most parts of Bangladesh, and so the country provides a highly favorable environment for the vector. *P. argentipes* is an indiscriminate feeder and feeds on whatever is in the vicinity. If given the choice, however, it prefers cow and human blood. Females may have up to four gonotrophic cycles. The sandflies are endophilic and

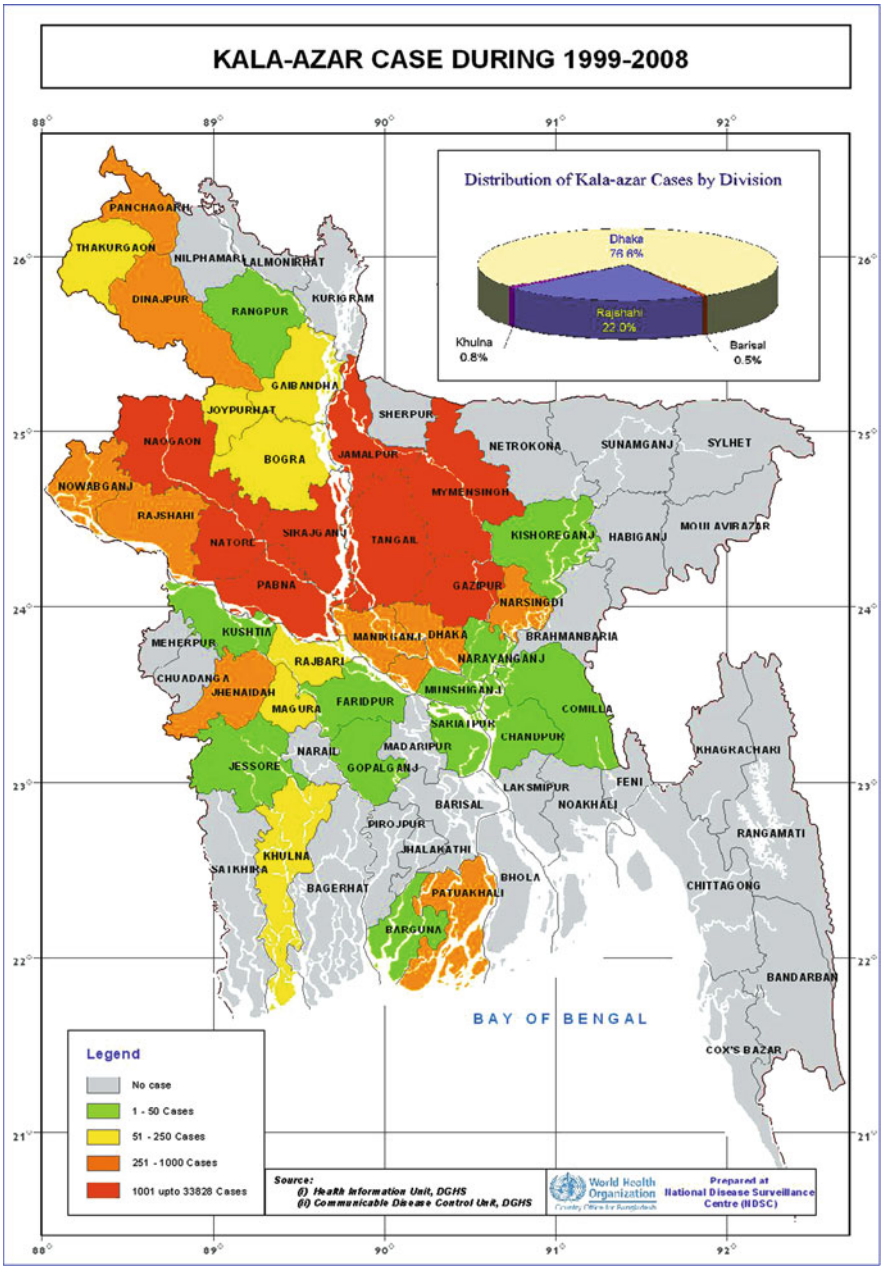

Fig. 1.3 Kala–azar endemic districts in Bangladesh with cases treated during the period 1999–2008

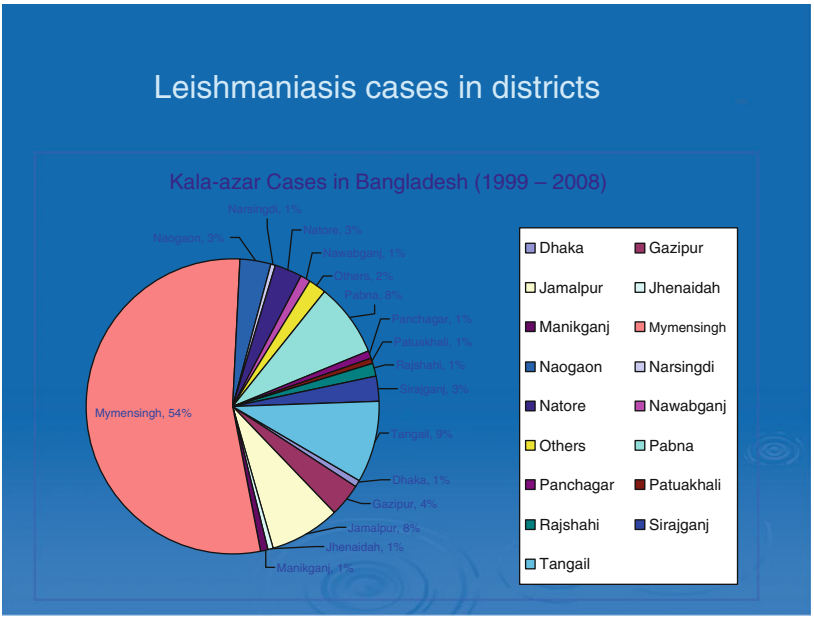

Fig. 1.4 District-wise distribution of Kala-azar cases in Bangladesh (1999–2008)

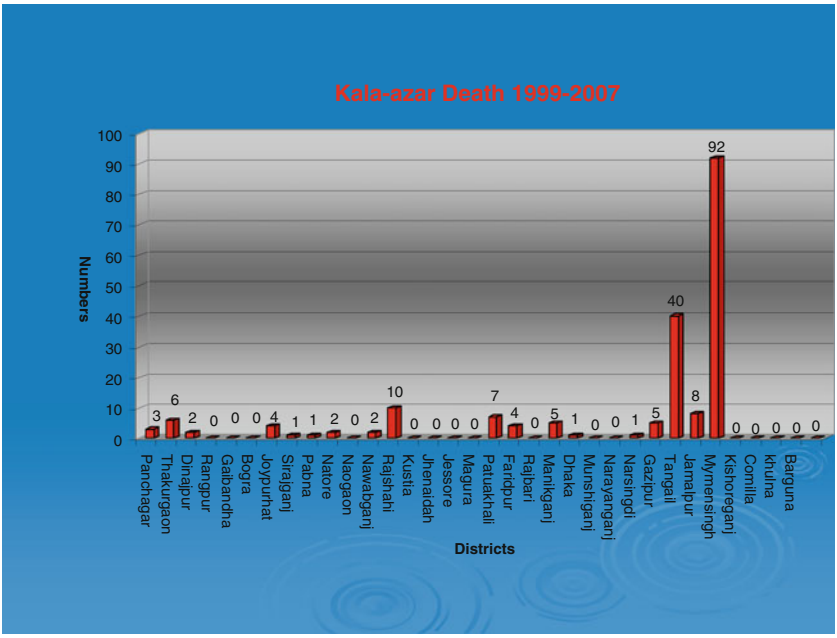

Fig. 1.5 District-wise distribution of death cases among Kala-azar patients reported during the period of 1999–2008

endophagic. They take short erratic hops and seldom rise more than 2 m (6 ft) above the ground. Dispersal is generally less than 300 m. *P. argentipes* has been found to be susceptible to DDT, malathion, and synthetic pyrethroids.

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Chapter 2
Pathology and Mechanism of Disease in Kala-Azar and Post-kala-azar Dermal Leishmaniasis

Moazzem Hossain and Kazi M. Jamil

Abstract The transmission of *Leishmania donovani* in humans is carried out via the bite of the infected female sandfly of the genus *Phlebotomus.* Flagellated promastigotes enter the skin, are engulfed by resident macrophages, and develop into amastigotes known as Leishman-Donovan bodies. These amastigotes are ingested by the sandfly along with a blood meal; they develop into promastigote form in the midgut of the sandfly, and become infective in about 9 days. The average incubation period in humans is 3–6 months, but it may be as long as 2 years. Kala-azar manifests with fever lasting more than 2 weeks, splenomegaly, anemia, weight loss, and increased pigmentation of the skin. Post-kala-azar dermal leishmaniasis is a sequel to kala-azar that usually develops 6 months to 5 years following an attack of untreated or incompletely cured visceral leishmaniasis.

Keywords Sand fly · Life cycle · Visceral leishmaniasis

Abbreviations

PKDL	Post-kala-azar dermal leishmaniasis
RE	Reticuloendothelial
VL	Visceral leishmaniasis

2.1 Life Cycle of *Leishmania Donovani* and Mode of Transmission of Kala-Azar

The natural transmission of *Leishmania donovani* in humans is carried out by the sandfly of the genus *Phlebotomus*. Through its bite, the infected female sandfly transmits the amastogote form of the parasite to the susceptible human.

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_2,

2.1.1 Life Cycle in Human Host

Flagellated promastigotes are transmitted to the human host through the bite of the infected sandfly. Some of the promastigotes are engulfed by the cells of the reticuloendothelial (RE) system, which makes them develop into amastogote form, becoming Leishman-Donovan bodies. Amastigotes multiply by binary fission until the host cell is packed with the parasites (with as many as 50–200 parasites in a single cell). The cells become enlarged and distended, and eventually they rupture, releasing the parasites back into the bloodstream, where they are either taken up by RE cells again or else they invade fresh cells, repeating the cycle. In this way, the entire RE system is progressively infected. In the bloodstream, some of the free amastigotes are phagocytosed by the neutrophilic granulocytes and monocytes. Blood-sucking sandflies ingest these free amastigotes along with their blood meal.

2.1.2 Life Cycle in Sandfly

Amastigotes ingested by the sandfly along with a blood meal develop into flagellated promastigote forms that multiply by binary fission in the midgut of the sandfly on the 3rd day after the blood meal. The flagellates tend to spread forward to the anterior part of the alimentary tract (pharynx and buccal cavity). A heavy pharyngeal infection of the sandfly is usually observed between the 6th and 9th day after its infected blood meal. In certain sandflies, heavy infection blocks the esophagus, and these sandflies regurgitate promastigotes from their buccal cavity into the puncture wound of the host when they bite again [1, 2, 3] Figs. 2.1 and 2.2.

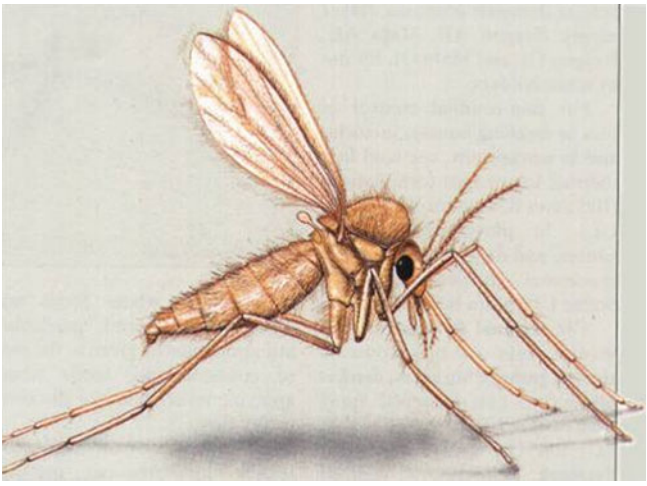

Fig. 2.1 Sandfly

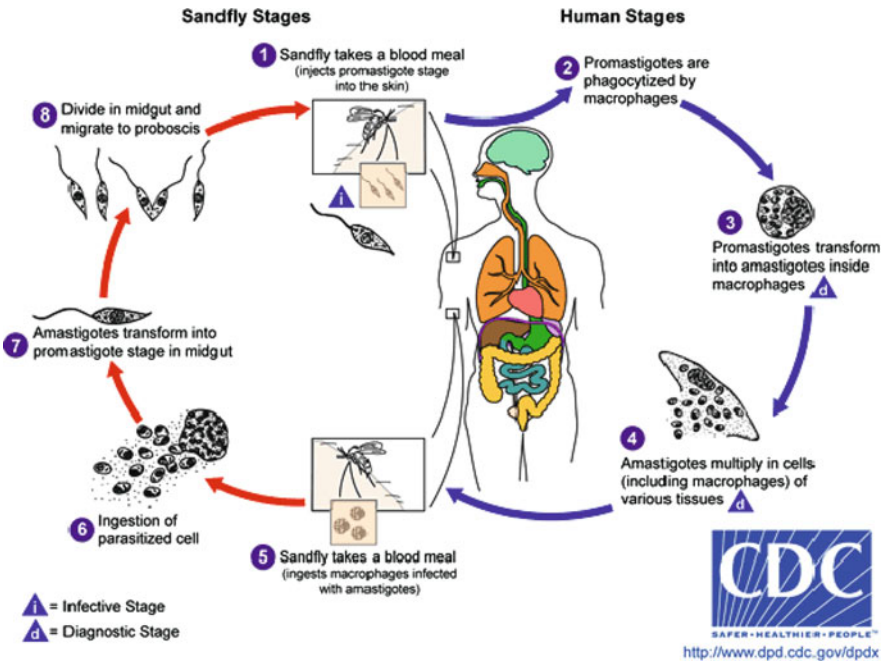

Fig. 2.2 Life cycle of *Leishmania donovani* obtained from CDC web site http://www.dpd.cdc.gov/dpdx/HTML/Leishmaniasis.htm

2.2 Pathogenesis and Pathophysiology of Kala-Azar

Promastigotes enter the punctured site of the host's skin through the bite of an infected sandfly, and mononuclear phagocytes (macrophages) and other white cells in the area are attracted to the area of entry. Some macrophages can kill the parasite directly, while others require prior stimulation before attaining the capability to destroy these parasites. To avoid being killed within the macrophages, the promastigotes transform themselves into amastigotes. Infected macrophages circulate in the blood or lymph and invade the viscera, especially the spleen, liver, and bone marrow. The skin may also become infected as a sequel to kala-azar, a condition known as post-kala-azar dermal leishmaniasis (PKDL), which is described below.

In acute visceral leishmaniasis (VL), there is a lack of cell-mediated immune response to leishmanial antigens, and therefore the parasites multiply rapidly. A humoral response occurs, with production of large amounts of non-specific polyclonal immunoglobulins, especially IgG, and specific antileishmanial antibodies are also produced. Patients who have recovered from VL are immune from re-infection, but relapses can occur.

2.3 Clinical Presentation of Kala-Azar

Kala-azar typically presents as fever which is usually insidious but may be associated with chills and rigor. The intensity of the fever decreases over time, and the patient may become afebrile for weeks or months, followed by a relapse of fever. Other clinical features of kala-azar include weight loss, anemia, abdominal swelling, bleeding manifestations (eg, epistaxis), and lymphadenopathy, among others.

2.4 Clinical Presentation of PKDL

In South Asia, PKDL usually develops 6 months to 5 years following an attack of VL, although up to 15% of PKDL cases occur without any preceding history of kala-azar. Skin lesions of various stages are found in PKDL, including macular, papular, nodular, or mixed types. The sensation over the lesions is preserved in PKDL, in contrast to leprosy, in which similar lesions show a loss of sensation. The skin lesions in PKDL usually do not ulcerate, and self-healing of the ulcers has also been reported Fig. 2.3.

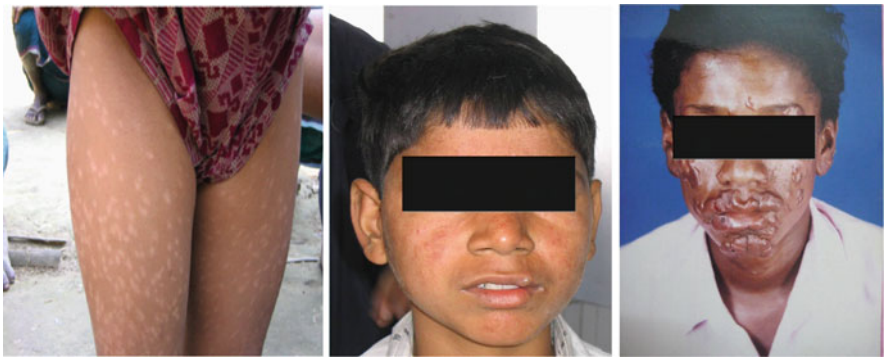

Fig. 2.3 Pictures of kala-azar and PKDL patients (**a**) macular type (**b**) papular type (**c**) nodular type

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Chapter 3
Diagnosis and Treatment of Kala-Azar and Post-kala-azar Dermal Leishmaniasis

Kazi M. Jamil and Moazzem Hossain

Abstract In South Asia, the diagnosis of kala-azar is based on a history of fever and a positive serological test for the presence of antibody against a 39 kDa recombinant antigen, the rK39 dipstick test. The clinical symptoms are characterized by prolonged and irregular fever often associated with chills and rigor, splenomegaly, lymphadenopathy, hepatomegaly, pancytopenia, progressive anemia, and weight loss. Kala-azar is almost always fatal if left untreated. Laboratory confirmation of the diagnosis requires demonstration of Leishman-Donovan bodies in smears of patients' splenic tissue, bone marrow, or lymph nodes. The serological test may remain positive for 2 years after completion of kala-azar treatment, and therefore is not useful to detect relapse or treatment failure. Diagnosis of post-kala-azar dermal leishmaniasis (PKDL) is typically based on previous history of kala-azar in a patient who presents with skin manifestations of macules, papules, or nodules without loss of sensation over these lesions. In India, PKDL develops within 2–3 years after treatment of kala-azar in 5–10% of cases. Many drugs are effective against kala-azar, including miltefosine, paromomycin, sodium stibogluconate (SSG), amphotericin B, and liposomal amphotericin B (AmBisome). Although monotherapy with one of these drugs has commonly been used for the treatment of kala-azar, a combination of these drugs is likely to be used in the near future when the results of ongoing clinical trials become available. Treatment options for PKDL are more limited, and long courses of treatment with SSG are still used.

Keywords Diagnosis · Kala-azar · Post-kala-azar dermal leishmaniasis · Treatment

Abbreviations

PKDL	Post-kala-azar dermal leishmaniasis
SSG	Sodium stibogluconate

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_3,

3.1 Diagnosis of Kala-Azar

A patient is considered to have kala-azar if the following criteria are met: (1) history of fever for 2 weeks or more; (2) living in an endemic area; and (3) a positive rK39 dipstick test [1]. Presence of splenomegaly, which is very commonly seen in kala-azar, may help in differentiating the condition from other causes of fever [2]. Anemia and a history of weight loss are also very common [2], and the presence of severe anemia may require hospitalization for blood transfusion. In South Asia, laboratory confirmation of kala-azar is not required for starting treatment, but parasitological diagnosis using splenic smears or bone marrow is needed to diagnose treatment failure or relapse. Although molecular diagnosis by polymerase chain reaction has been reported, its use remains mostly confined to research purposes only.

3.2 Diagnosis of Post-kala-azar Dermal Leishmaniasis

In India, post-kala-azar dermal leishmaniasis (PKDL) develops within 2–3 years after treatment for kala-azar in 5–10% of cases [3]. A patient is diagnosed as having PKDL if the following features are present: (1) history of treatment for kala-azar (present in 85% cases); (2) presence of macules, papules, or nodules without any loss of sensation over the lesions; and (3) a positive rK39 dipstick test. Demonstration of the parasite by skin biopsy or skin smear is recommended before initiating therapy.

3.3 Treatment of Kala-Azar

The first-line drugs for treatment of kala-azar include miltefosine and paromomycin, and the second-line drugs are amphotericin B, liposomal amphotericin B (AmBisome; Gilead Sciences, Foster City, CA, USA), and sodium stibogluconate (SSG) (Table 3.1) [4]. Each of these drugs has its merits and demerits. Miltefosine is the only oral drug on the market for the treatment of kala-azar. It is contraindicated for pregnant and lactating women, and in children below 2 years of age. Women of child-bearing age must use contraception during treatment and for 3 months after treatment with miltefosine. Paromomycin treatment requires painful intramuscular injections for 21 days. Its side effects include minimal ototoxicity and nephrotoxicity, both of which are reversible. It should be avoided in patients with hemoglobin of <5 g/dL. It is the cheapest drug available for the treatment of kala-azar.

Concerning the second-line drugs, amphotericin B has side effects that include fever with chills and rigor. Hypokalemia, nephropathy, and myocarditis may occur occasionally. The infusion should be given slowly over a period of 6 h. Patients

Table 3.1 Drugs used for treatment of kala-azar

Drug name	Dose schedule
Miltefosine	Adults (>12 years) weighing >25 kg: 50 mg cap twice daily, given after food for 28 days Adults (>12 years) weighing <25 kg: 50 mg cap once daily, given after food for 28 days Children (2–11 years old): 2.5 mg/kg body wt in two divided doses, given after food for 28 days [*Are persons older than 11 considered adults?*]
Paromomycin	Intramuscular injection in a dose of 15 mg/kg/day for 21 days
Amphotericin B deoxycholate	Intravenous infusion (in 5% dextrose solution) at a dose of 1 mg/kg for 15–20 days
Liposomal amphotericin B	Intravenous infusion at a dose of 3 mg/kg for 5 days or 5 mg/kg for 3 days (total dose of 15 mg/kg)
SSG	Intramuscular injection in a dose of 20 mg/kg/day for 30 days, not exceeding a daily dose of 850 mg

must remain under close observation for the monitoring of side effects. Liposomal amphotericin B is a very safe and effective drug with minimal side effects. It is also the most expensive of all the drugs available for the treatment of kala-azar. Finally, SSG is a cardiotoxic drug and is associated with high mortality due to cumulative toxicity. It may be used in pregnant and lactating women to avoid teratogenic drugs like miltefosine, if liposomal amphotericin B is not available.

3.4 Treatment of PKDL

The treatment of PKDL remains a challenge for the kala-azar elimination program, since there are no satisfactory regimens available yet. Amphotericin B or liposomal amphotericin B is recommended if these drugs are available. However, SSG still remains the mainstay of therapy for PKDL in most endemic areas in Bangladesh. The drugs are administered in the following doses for one cycle, and the course is completed after six cycles of treatment (Table 3.2).

Table 3.2 Treatment regimens for PKDL (for each cycle)

Drug name	Dose schedule
Amphotericin B deoxycholate	Intravenous infusion (in 5% dextrose solution) at a dose of 1 mg/kg for 15–20 days
Liposomal amphotericin B	Intravenous infusion at a dose of 3 mg/kg for 5 days or 5 mg/kg for 3 days (total dose of 15 mg/kg)
SSG	Intramuscular injection in a dose of 20 mg/kg/day for 20 days, not exceeding a daily dose of 850 mg

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Part II
Therapeutic Strategy to Deal with Emergence of Drug Resistance

Chapter 4
Epidemiology of Drug-Resistant Kala-Azar in India and Neighboring Countries

T.K. Jha

Abstract Sodium stibogluconate (SSG), a pentavalent antimonial compound administered parenterally at a daily dose of 10 mg/kg for 10 days has been the first-line treatment for kala-azar on the Indian subcontinent for more than 70 years, with a cure rate of > 90%. However, in the 1970s and early 1980s this regimen failed to cure an appreciable percentage of kala-azar cases from the hyper endemic Indian state of Bihar. In 1982 and 1990, the World Health Organization recommended a higher and prolonged dosage regimen for use in resistant zones of Indian kala-azar. But by the year 2000, even these stronger treatments failed to cure as many as 60% of cases. Even in the districts of Nepal bordering Bihar, the efficacy rate declined to 59.9%. Diamidine compounds were used extensively in SSG-resistant cases in Bihar between the late 1970s and 2003. The cure rate when 10–12 injections were given remained at 98.2% until the late 1980s. However, by the 1990s longer treatment was required, and the efficacy rate declined while the incidence of serious toxicities increased. Thus, the use of this drug was discontinued. Among the drugs administered orally, miltefosine was found highly effective in the treatment of kala-azar in different phases of trials in India, with a cure rate of 94%. But the cure rate declined to 82–87% in outpatients. Paromomycin, administered intramuscularly for 21 days, was found to have a cure rate of 94% in different phases of trials in India. It has been registered in India since August 2006, and is a potential first-line drug. Amphotericin B or its liposomal compound (AmBisome) remains the most potent drug with a cure rate of 98–100% in all resistant cases of kala-azar. To counter the problem of drug resistance, particularly in patients of kala-azar co-infected with HIV, a combination therapy of two potent drugs or alternatively two courses of AmBisome is recommended.

Keywords Drug resistance · Indian subcontinent · Kala-azar · Unresponsiveness

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_4,

Abbreviations

AIDS	Acquired immune deficiency syndrome
DDT	Dichlorodiphenyltrichloroethane
HIV	Human immunodeficiency virus
IFNy	Interferon gamma
SAG	Sodium antimony gluconate
SSG	Sodium stibogluconate
VL	Visceral leishmaniasis
WHO	World Health Organization

4.1 Introduction

The global incidence of kala-azar or visceral leishmaniasis (VL) is at approximately 500,000 new cases per year. Ninety percent of these cases are from India and the neighboring countries of Bangladesh and Nepal on the Indian subcontinent, as well as Sudan and Brazil. Kala-azar practically disappeared from the Indian subcontinent following the program of dichlorodiphenyl-trichloroethane (DDT) spraying to eradicate malaria between the 1950s and the late 1960s. The resurgence of this disease began in the mid-1970s in India and in the late 1970s in Bangladesh, where in the 1980s an outbreak occurred in the Pabna district. The first outbreak of kala-azar was reported in 1980 from the plains of Nepal bordering the northern districts of the Indian state of Bihar. Fifty percent of the cases reported from India are from Bihar. Twenty-eight of 37 districts of Bihar are affected and 200,000 deaths from VL have been reported from Bihar since 1977 [1, 2]. One hundred nine districts on the Indian subcontinent are affected: 45 in Bangladesh, 52 in India, and 12 in Nepal. Total burden of the disease is 21 cases per 10,000 population in these three countries.

4.1.1 History of Kala-Azar on the Indian Subcontinent

Kala-azar – the word "kala" means black, referring to the black pigmentation of the skin, and "azar" means prolonged fever or ailment – has been known to occur on the Indian subcontinent for more than 180 years. The first description of an epidemic of this disease was reported in 1824–1825 in Jessore (now Bangladesh) and involved approximately 7,500 fatalities. The disease spread to Bengal between 1830 and 1850, and was carried via river travel to the Brahmaputra valley in Assam in 1875. In 1872, it traveled along the Ganges River from Bengal to the plains of Bihar, where the disease was given the name kala-dukh (black disease) [3]. An epidemic of the disease involving 9,400 cases was reported in Bihar in 1895 (Fig. 4.1) [3]. The resurgence of kala-azar began in the mid-1970s. In a 1977 epidemic, 100,000 cases were reported. A major epidemic occurred in Bihar during the period 1991–1992, with 250,000 cases and a mortality rate of 35% [4].

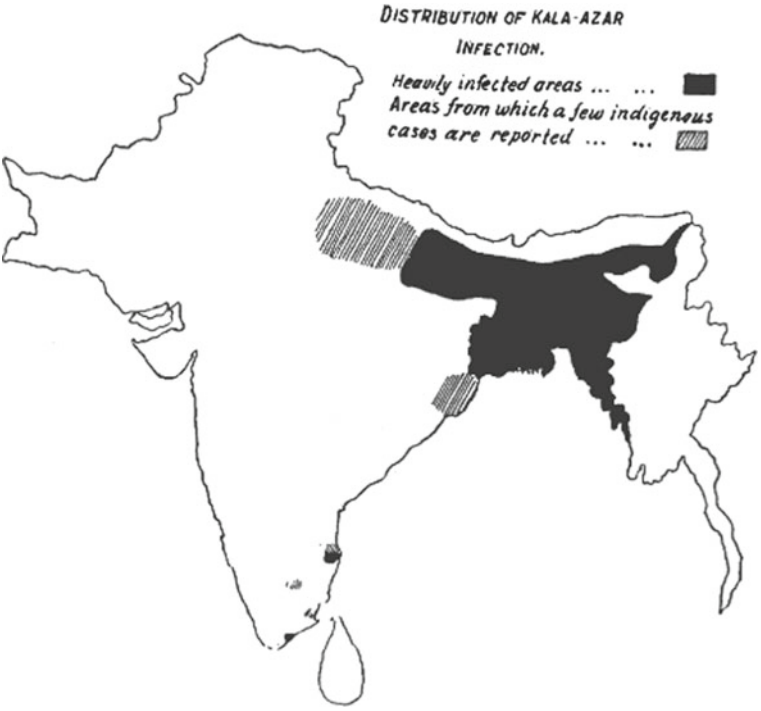

Fig. 4.1 Map of Indian subcontinent, 1903 (Source: Indian Med Gazette, May 1947, p. 281)

4.1.2 Incidence of Kala-Azar in Bangladesh

The prevalence of kala-azar was substantially reduced in India and Bangladesh following DDT spraying for malaria during the 1960s [5]. Sporadic cases were reported in the late 1970s, and an outbreak occurred in the Pabna district in 1980 [6]. Cases of kala-azar have been reported every year since then. Thirty-four of 64 districts are affected, with 90% of these cases found in a mere 10 districts (Fig. 4.2). Three thousand nine hundred and sixty-five cases were reported in 1994, and 8,920 cases in 2004. It is estimated that the actual number of cases is five times the number reported [7].

4.1.3 Incidence of Kala-Azar in Nepal

Twelve districts of Nepal are endemic for kala-azar (Fig. 4.3), with a population of 5.5 million at risk. Since 1980, 14,685 cases and 215 deaths from this disease have been reported, and this figure may be lower than the actual incidence. The case incidence is about 43 per 100,000 population at risk, and the fatality rate ranges from 0.84 to 1.75% [8].

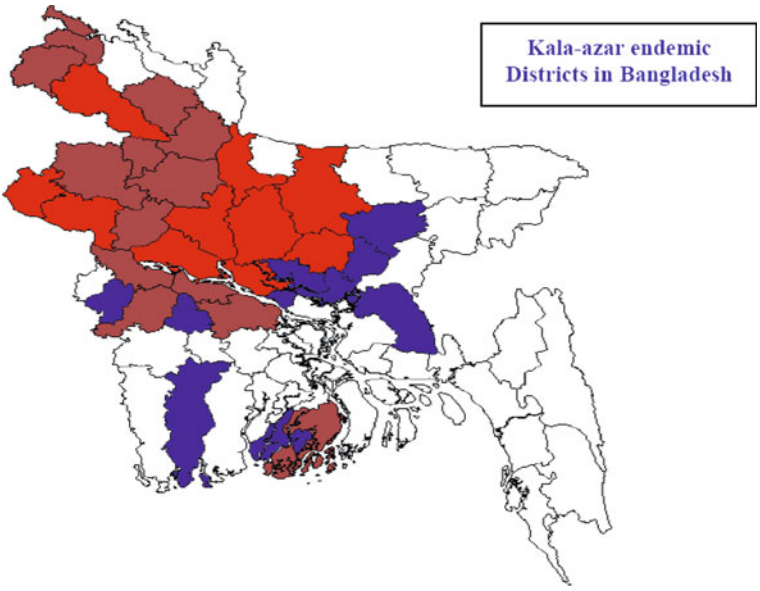

Fig. 4.2 Map of Bangladesh (Source: http://www.searo.who.int/en/Section10/Section2163_13267.htm)

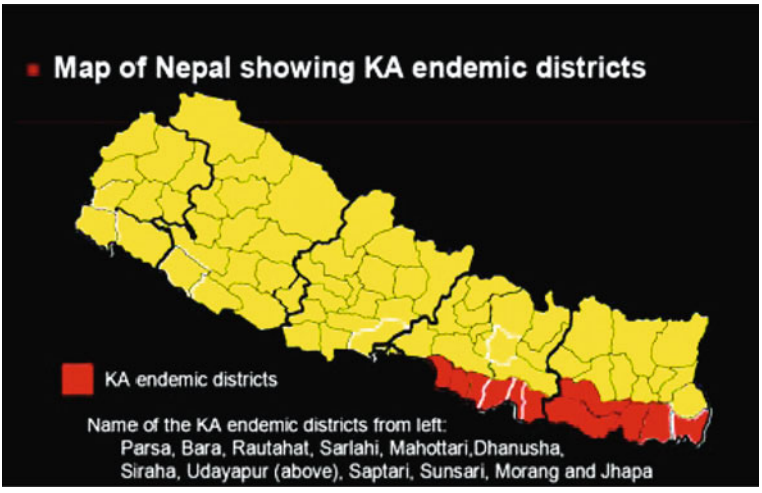

Fig. 4.3 Map of kala-azar trends from 1987 to 2006 (Source: http://www.searo.who.int/en/Section10/Section2163_13267.htm)

4.1.4 The Current Scenario on the Indian Subcontinent

Historically, the VL epidemic cycle repeats regularly every 15–20 years [9]. On the Indian subcontinent, about 200 million people are at risk for VL. It is estimated

that 25,000–40,000 cases and 200–300 deaths occur yearly in the three affected countries. These figures are considered to be grossly underestimated. One hundred nine districts on the Indian subcontinent are affected: 45 in Bangladesh, 52 in India, and 12 in Nepal, with an increasing trend in Nepal and Bangladesh. The estimated number of VL cases in Bangladesh, India, and Nepal are 136,500, 270,900, and 12,600, respectively. The total burden of the disease on the subcontinent is 21 cases per 10,000 population [10].

4.2 History of the Efficacy of Antileishmanial Drugs on the Indian Subcontinent

4.2.1 Sodium Stibogluconate (SSG) Resistance in Nepal

A prospective study was conducted in a tertiary-level hospital located in south-eastern Nepal from July 1999 to January 2001 on newly parasitologically positive cases of VL. One hundred twenty cases were enrolled in the study, of which 110 patients completed SSG therapy. SSG was used at a dosage of 20 mg/kg daily for 30 days, which was extended to 40 days for patients who were still parasitologically positive. Of the 110 patients completing therapy, the definite cure rate at 6-month follow-up was 90% (99 patients), and 10% had treatment failure. However, a definite cure rate of only 76% was achieved in those patients coming from the plains of Nepal bordering North Bihar. Four patients (3.3%) died, and overall cardiac toxicity was observed in 3.3% [11].

In another study conducted in a tertiary hospital in Nepal between 1999 and 2004, 659 VL patients were enrolled. Five hundred twenty-six cases came from districts outside the SSG-resistant zone of North Bihar. One hundred thirty-three cases came from the plains of Nepal bordering known SSG-resistant districts in North Bihar. Of these, 480 and 133 cases, respectively, were assessed. Final cure rates of 86.1 and 59.9%, respectively, were achieved [12].

4.2.2 Efficacy of Miltefosine in Nepal

A phase 4 trial of miltefosine in Nepal was conducted during September 2003–March 2004 on 125 patients, of which 122 completed treatment. A final cure rate of 88% (105 of 119 cases) was achieved (unpublished data).

4.2.3 Efficacy of SSG in Bangladesh

No data from any controlled trial testing the effectiveness of SSG have been reported so far from Bangladesh. There is a shortage of the standard preparation of SSG in this country. However, as per official recommendations, SSG is used at a dose of 20 mg/kg daily for 28 days. In one study (personal communication), 148 patients were enrolled, of which 144 had an initial clinical cure at the end of treatment. Four

patients died during treatment. The final cure rate at 6-month follow-up could not be ascertained. A phase 4 study of miltefosine was conducted in Bangladesh by the Special Programme for Research and Training in Tropical Diseases of the World Health Organization (WHO) in cooperation with Zentaris (Germany). The results are not yet published (personal communication).

4.2.4 Changing Response to Different Antileishmanial Agents in India

Pentavalent antimonials have been the main first-line treatment for all forms of leishmaniasis, including VL. After its discovery by Schmidt, Kikuth, and others, antimony gluconate (Solustibosan R) was synthesized in 1937. Several pentavalent antimonials have been synthesized since then and are available in the world market. Meglumine antimoniate is marketed as Glucantine and Prostib. SSG or sodium antimony gluconate (SAG) is available commercially as Pentostam, Solustibosan, and Stibanate. Generic sodium antimony gluconate (Albert David, Calcutta, India) is 20 times cheaper than other products, costing US$13 versus US$200 per patient. There is some variation in the effectiveness of SSG in different states in India. Patients from eastern Uttar Pradesh are still responsive to SSG [13]. In the Dumka district of Jharkhand, where kala-azar is common, SSG remains effective, as well.

SSG has been in use in India for treating kala-azar since 1940. Used in the dose of 10 mg/kg daily for 10 days, administered parenterally (intramuscularly or intravenously), it has proved effective in curing 95% of cases. Thus, a disease with over 90% mortality in untreated patients can be cured in 95% of them [3]. Until 1970, SSG used in the conventional dosage was effective in curing more than 90% of cases of Indian kala-azar. In an earlier resurgence of kala-azar, which assumed epidemic proportions by 1977, an estimated 250,000 patients were affected in Bihar. Thirty percent of cases were unresponsive to SSG [14].

During the late 1970s and early 1980s, different healthcare workers used different regimens of SSG to counter the problem of unresponsiveness before the pharmacokinetic studies of SSG were conducted (Table 4.1) [15–17]. A pharmacokinetic study of SSG was done in 1980. It was observed that the use of SSG can be safely prolonged up to 60 days when administered in a daily dose of 10 mg/kg. Tolerance in children was found to be better than in adults [18]. In 1982, WHO recommended SSG at a daily dose of 20 mg/kg (maximum dose of 850 mg) for 20–30 days in new cases and for 40–60 days in relapse cases. Subsequently, WHO in 1990 recommended SSG at a daily dose of 20 mg/kg for 30 days [19]. Changes in the response to the WHO regimen were observed in Bihar by different healthcare workers (Table 4.2) [20–25]. However, unresponsiveness to the higher and prolonged dosage schedule recommended by WHO caused treatment failure in up to 60% of cases of kala-azar from the hyper endemic districts of North Bihar (Fig. 4.4).

Drug-induced toxicities such as myocarditis, pancreatitis, renal failure, and deaths following WHO's new SSG regimen were reported. Even before this, there

Table 4.1 Efficacy of SSG in different dosage and duration between 1975 and 1981

Healthcare worker	Dose (mg/kg per day)	Duration in day	Number of courses	Number of cases	Number of unresponsiveness (%)
Jha (1975–1976) [15]	10	10	1	200	17
Jha (1977–1980)[a]	10	10	3	520	30
Thakur (1980–1984) [16]	10	20	1	126	9
Jha (1980–1981) [17]	10 Adult 20 Child	30-Fresh 42-Slow responder 60-Relapse	1	90	1.1

[a]Unpublished data.

Table 4.2 Changing response to SSG used in WHO regimen

Healthcare worker	Dose (mg/kg per day)	Duration in day	Number of courses	Number of case	Number of unresponsiveness (%)
Thakur (1984–1987) [20]	20	40	1	371	8
Jha (1989) [21]	20	30	1	252	26.1
Jha (1991) [22]	20	30	1	32	25
Jha et al. 1996 BMJ [23]	20	30	1	30	36.7
Thakur et al. 1998 [24]	20	30	1	80	54
Sundar et al. [25]	20	30	1	184	60

was controversy over whether the treatment failure was due to immunological failure or drug resistance. However, a 100% treatment failure after SSG was administered in the WHO-recommended dosage, was observed in two rural villages located in North Bihar. A total of 241 cases were found in the two villages [26]. Pharmacological failure was suggested to be the likely cause of treatment failure [27].

In 1991, Jha defined the therapeutic response zones of North Bihar (Fig. 4.5) [26].

4.2.5 Changing Response to Diamidine Compounds

Among diamidine compounds, pentamidine isethionate (May and Baker) and pentamidine demethane sulphonate (Lomidine-Specia, France) have been used in SSG-unresponsive cases of kala-azar from the late 1970s to 2003 as a second-line treatment. In one study, 82 patients who were unresponsive to SSG were treated with pentamidine isetheonate at a dose of 4 mg/kg for 10–12 intravenous injections on alternate days, with a cure rate of 98.2% at 6-month follow-up. Two of the 82 patients died of sudden cardiac arrest and 4.81% ($n = 4$) developed a condition resembling diabetes mellitus [28].

Fig. 4.4 Map of Bihar showing therapeutic status of SSG in Bihar

Fig. 4.5 Map of Bihar showing endemic zones

In another study, 233 SSG-resistant cases were treated with pentamidine at a dose of 4 mg/kg administered intramuscularly or intravenously on alternate days. One hundred seventy-four cases received pentamidine isetheonate, while 59 were treated with pentamidine methane sulphonate (Lomidine). Clinical and parasitological evaluation was done every 10th day until two successive bone-marrow or splenic aspirates were negative for parasites. Eighteen to 20 injections were required for an initial cure. A full cure was achieved in 75.2% of the cases treated with pentamidine isetheonate, while a cure rate of 100% was observed in the Lomidine group. Drug-induced diabetes mellitus was observed in 1.14 and 18.4%, respectively. An overall cure rate of 81.5% and relapse rate of 2.08% were observed. Mortality rates of 0.57 and 9.23% were observed in the two groups [29].

In another randomized study, pentamidine isetheonate was used as a monotherapy at a dose of 4 mg/kg administered parenterally in three injections per week until a parasitological cure was achieved. Thirty-three or more injections were required to achieve a cure rate of 98%. However, toxicities such as hyperglycemia were observed in 10% of cases (6% reversible, 4% irreversible), and cardiac toxicity was also observed [30].

Both of the diamidine compounds are imported, expensive, and highly toxic. Drug-induced irreversible insulin-dependent diabetes is reported in 5–12% of cases. Cardiac toxicities leading to fatal arrhythmias have been observed. This drug is no longer used to treat Indian kala-azar due to the declining response, serious toxicities, and high cost [31].

4.2.6 Amphotericin B

At present, amphotericin B is used extensively in Bihar for all cases of SSG-unresponsive cases of kala-azar. In some of the hyper endemic districts of North Bihar where between 30 and 60% of cases are SSG-unresponsive, it is being used as the first line of therapy. It is used at a dose of 1 mg/kg administered intravenously with 5% dextrose on alternate days for 15 injections. A cure rate of 98–100% is achieved at 6-month follow-up. This is a very effective but costly treatment, requiring hospitalization, and febrile reactions are very common. Monitoring for nephrotoxicity and checking serum electrolytes for hypokalemia is required.

4.2.7 Liposomal Amphotericin B (AmBisome)

There are three liposomal preparations: liposomal amphotericin B sold as AmBisome (Gilead Sciences; Dimas, California, USA); amphotericin B Lipid complex sold as Abelcet (Enzon Pharma; Fairfield, New Jersey, USA); and amphotericin B colloidal dispersion, sold as Amphotec (Intermune; Brishane, California, USA).

AmBisome used to treat resistant VL in a cumulative dose of 10–15 mg/kg cured 90–100% of cases. The duration of therapy could be reduced to 2–5 days [32]. A total dose of 20 mg/kg, divided into four or five doses given intravenously on day 1, day 3, day 5, and day 7 resulted in a 100% cure rate when used as a rescue drug in resistant cases of VL in different drug trials (unpublished data). At present, this is the most effective drug for kala-azar that can be used in all resistant cases. However, it is too costly (one 50 mg vial of AmBisome costs about US$135).

4.2.8 Paromomycin (Aminosidine)

Paromomycin is an aminoglycoside found to have a potent antileishmanial action. Previously, it was tried in combination with SSG to improve cure rates [33]. In a phase 2 randomized controlled trial, paromomycin was used at a daily dose of 16 or 20 mg/kg given intramuscularly for 21 days. A cure rate of 93 and 97%, respectively, was achieved at 6-month follow-up [34]. In a phase 3 multicenter double-blind paromomycin study involving 500 cases of Indian kala-azar, a cure rate of 94.6% was achieved at 6-month follow-up [35]. A phase 4 trial has recently been conducted at 17 sites. The results of this study have not yet been released. Paromomycin is a potential first line of treatment for kala-azar on the Indian subcontinent when used as a monotherapy. Problems of relapse and treatment failure can be anticipated as with other aminoglycosides when used as a monotherapy.

4.2.9 Status of Oral Treatments for Kala-Azar

Allopurinol and ketokonazole have been tried in cases of Indian kala-azar both singly and in combination with SSG without much success [36, 37].

4.2.10 Miltefosine as a Treatment for Kala-Azar

Miltefosine is an active alkylphospholipid derivative found to have potent antileishmanial activity. It has undergone several clinical trials, including phase 1/2 [38]. phase 2 [39], and phase 3 [40]. in adults, phase 3 in children [41], and phase 4 in both children and adults [42]. Altogether more than 1,700 new and resistant cases of kala-azar in adults and children were enrolled in different multicenter trials in Bihar [42]. A final cure rate of 94% at 6-month follow-up was achieved. Miltefosine is used at a dose of 50 mg twice daily (100 mg/day) for 28 days in patients weighing more than 25 kg; 50 mg daily for 28 days for children weighing 25 kg; and 2.5 mg/kg daily for 28 days for children aged 2–11. However, the cure rate dropped to about 82–87% in phase 4 trials when used in outpatients, due to noncompliance related to gastrointestinal disturbance.

Miltefosine has a long half-life of about 150 h, which may create problems of relapse and treatment failure in the future. It is not recommended for use in females of child-bearing age unless they use contraception during and for 2 months after the

end of treatment. Of 18,462 VL cases seen between 1975 and 2009 at the Kalazar Research Centre (Brahmpura, Muzaffarpur, Bihar, India), 18.56% of cases were females of child-bearing age (unpublished data). This female population is likely to be excluded from treatment with miltefosine.

4.2.11 Sitamaquin (WR 6026)

Sitamaquin is a primaquin analogue with effective antileihsmanial activity. In a multicenter phase 2 dose-finding study on 120 Indian kala-azar patients, sitamaquin was used at a dose of 1.5, 1.75, 2.0, and 2.5 mg/kg daily for 28 days. A final cure rate of 81, 89, 100, and 80%, respectively, was achieved. However, 5% of patients had nephrotoxicity [43]. Sitamaquin can potentially be used as an oral drug in combination with another first-line antileishmanial agent.

4.3 Combination Therapy for Indian Kala-Azar

Combining one potent antileihsmanial drug with a short half-life and another drug with a long half-life may improve the efficacy and cure rate in VL as with tuberculosis or leprosy. The second drug may have a synergistic or additive effect without having an adverse drug interaction. SSG has been used with Th1-activating cytokine (interferon-gamma [IFN-γ]) as a form of immunochemotherapy to treat Indian kala-azar. IFN-γ at a daily dose of 100 $\mu g/m^2$ for 30 days combined with SSG at a daily dose of 20 mg/kg for 30 days was used, and full cure was achieved in only 49% of cases [44]. In India and the plains of Nepal, where unresponsiveness to SSG is very high, this combination therapy is ineffective. In 1995, when SSG was used in combination with allopurinol or ketoconazole to treat Indian kala-azar, no significant increase in efficacy was observed [37].

4.4 Combination Therapy in Kala-Azar Associated with HIV/AIDS

There is increasing incidence of coinfection of HIV/AIDS with kala-azar in India, Bangladesh, and Nepal. In 1994, not a single case of HIV/AIDS associated with kala-azar was reported [45]. In 1997, three cases of coinfection were reported [46–48].

In a study at the Kalazar Research Centre, 61 cases of coinfection were reported out of 573 cases of HIV/AIDS during the period from January 1995 to January 2009. A combination of amphotericin B 1 mg/kg daily for 20 days followed by miltefosine 100 mg daily for 28 days was used in three cases without relapse at 2-year follow-up. Those who received amphotericin B or miltefosine as a monotherapy did not respond and died within 6 months. All cases received antiretroviral therapy, as well [49]. Two cases of coinfection were treated with AmBisome at a total dose of 20 mg/kg divided into four doses. One relapsed within 3 months and required

another course of drug therapy. Both of them achieved full cure from VL at 2 year follow-up.

At present, combination therapy using a single low dose of AmBisome followed by 14 days of miltefosine, and another combination using intramuscular paromomycin along with 14 days of miltefosine are under trial to reduce the dose and duration of therapy, to prevent drug resistance and to improve the cure rate for Indian kala-azar.

4.5 Conclusion

Unresponsiveness to SSG has been increasing in India and the plains of Nepal. In Bangladesh, the status of SSG efficacy is not well known. SSG as a first-line therapy is not recommended for the kala-azar elimination program in India and the neighboring countries such as Nepal and Bangladesh. In 2005, a memorandum of understanding was signed by the governments of India, Nepal, and Bangladesh along with the WHO Regional Office for South-East Asia in New Delhi and the WHO Special Programme for Research and Training in Tropical Diseases in Geneva, Switzerland, calling for the elimination of kala-azar by the year 2015. The present burden of VL in these countries is 21 cases per 10,000 population. The target is to reduce this statistic to one case per 10,000 population by 2015. At present, miltefosine is recommended as a first-line therapy for the elimination program. It would be ideal to use it as a direct observed treatment to counter the problem of noncompliance. An injectable Depo-Provera contraceptive preparation should be given a few days before starting miltefosine in females of child-bearing age on the Indian subcontinent to avoid any teratogenic complications in the case of patients getting pregnant during and 2 months after therapy. However, discussion is still ongoing whether a single intravenous dose of 10 mg/kg of AmBisome can be considered as a first line of therapy in the elimination program in South-East Asia.

Acknowledgments Gratitude is expressed to Mr. Sarvajeet Kumar, Clinical Research Coordinator, Kalazar Research Centre, Brahmpura, Muzaffarpur, for secretarial assistance, and also to Dr. C. P. N. Thakur, Director, Pathological Research Lab, for reviewing the manuscript.

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Chapter 5
A Therapeutic Strategy for Treating Visceral Leishmaniasis in Regions with Drug Resistance

Shyam Sundar and Dipti Agarwal

Abstract Visceral leishmaniasis (VL) affects 500,000 people annually worldwide, and Bihar alone accounts for approximately 45% of that burden. For last two decades there has been a steady decline in response to pentavalent antimonial (Sb^v), the drug that has been used for treating VL for seven decades. Oral miltefosine has been chosen as an alternative drug for use in the kala-azar elimination program in India, Nepal, and Bangladesh. There are only four approved antileishmanial drugs: Sb^v, miltefosine, paromomycin, and amphotericin B and its lipid formulations. Except for liposomal amphotericin B, all the other drugs have to be administered for 21–30 days and have frequent side effects, leading to non-compliance and early discontinuation of treatment. These factors, along with the intrinsic characteristics of the drugs (e.g., a long half-life in the case of miltefosine), are conducive to the development of drug resistance. Thus, alternative strategies must be developed to prolong the effective life span of these drugs, such as the use of single-dose liposomal amphotericin B, or directly observed therapy when longer-duration treatments are used. Finally, combination therapy with multiple drugs should be implemented as early as possible, because these are likely to shorten the duration of therapy, improve compliance, and decrease both toxicity and cost of treatment.

Keywords Amphotericin B · Antimony · Miltefosine · Paromomycin · Visceral leishmaniasis

Abbreviations

AIDS	Acquired immune deficiency syndrome
HIV	Human immunodeficiency virus
Sb^v	Pentavalent antimonial
VL	Visceral leishmaniasis
WHO	World Health Organization

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_5,

5.1 Introduction

Visceral leishmaniasis (VL) is the systemic and disseminated form of a disease also known as kala-azar. This syndrome is typically characterized by fever (often with chills and rigor) splenomegaly, hypergammaglobulinaemia, and pancytopenia. Once clinical VL sets in, patients become emaciated and prone to develop secondary infections such as malaria, pneumonia, tuberculosis, and amebic or bacillary dysentery. VL is a lethal form of leishmanisis that is uniformly fatal unless treated. The incidence of VL is 500,000 cases per year. Ninety percent of the annual global burden of VL cases occurs in India, Nepal, Bangladesh, and Brazil. In these countries, VL is endemic, and epidemics are quite frequent, which leads to considerable mortality. It thus is a significant public health problem. In India, about 100,000 cases of VL are estimated to occur annually, with the state of Bihar accounting for > 90% of these cases.

Unfortunately, in Northern Bihar in India, > 50% of patients are refractory to pentavalent antimonial (Sb^v) therapy, and thus alternative drugs have to be used [1, 2]. The failure of Sb^v is attributed to its widespread misuse in this anthroponotic focus with intense transmission [3]. A similar potential for resistance exists in East Africa, especially in Sudan, another anthroponotic focus of VL with intense transmission, where poverty, illiteracy, and poor healthcare facilities portend the misuse of the drug and consequent emergence of resistance [3]. Resistance develops as the epidemic turns endemic in foci where Sb^v has been used as a monotherapy for long periods, often with poor supervision and compliance [4]. In other parts of the world, Sb^v continues to be effective [5]. The antileishmanial drugs discussed in the following sections are available for use to plan a strategy for treatment in regions with drug resistance.

5.2 Antileishmanial Drugs

The treatment of VL has evolved significantly over time as a consequence of emerging resistance patterns and newer drug delivery systems.

5.2.1 Sodium Stibogluconate

Urea stibamate was the first antimonial drug, introduced over 70 years ago. It was replaced in the 1950s by sodium stibogluconate, which became the first-line treatment for VL. Initially, the drug was used at very low doses (e.g., 10 mg/kg per day for 6–10 days). For more than seven decades, Sb^v compounds have been used for the treatment of all forms of leishmaniasis. The drug was cheap, and, at that time, effective and well-tolerated. Then treatment failures started to occur, and a routine of gradually increasing the dose and duration of therapy began, in what has proven to be an unsuccessful attempt at catching up with the resistance. As a result

of the increased doses, adverse events became common, including arthralgia, myalgia, and raised hepatic transaminases. In a significant proportion of patients, severe toxicity such as chemical pancreatitis, especially in patients coinfected with human immunodeficiency virus (HIV) [6] and cardiotoxicity, manifested by concave ST-segment elevation, prolongation of the QT interval to >0.5 millisecond, ventricular ectopics, runs of ventricular tachycardia, torsades de pointes, ventricular fibrillation, and sudden death. With substandard Sb^v compounds, cardiotoxicity resulting in death occurs very frequently.

5.2.2 Amphotericin B

Amphotericin B is a polyene antibiotic isolated from *Streptomyces nodosus* that is used as a first-line drug in regions with high levels of unresponsiveness to Sb^v.[7], [8] While it is conventionally an antifungal drug, its antileishmanial activity was first shown in the early 1960s. In VL, amphotericin B should be used in doses of 0.75–1 mg/kg body weight on alternate days for at least 15 injections or until a cure is achieved. The presence of fungus-like sterols as the primary demethylated sterols (ergosterol) provides the rationale for the antileishmanial activity of amphotericin B. It is a polyene antibiotic that has high affinity to these ergosterol-like sterols in the cell membrane, inhibiting its incorporation into the membrane-forming micropores and leading to increased membrane permeability and ultimately the killing of the *Leishmania*. Amphotericin B infusions can cause renal dysfunction, hypokalemia, hepatic dysfunction, bone marrow suppression, and myocarditis, any of which can be fatal. Fever with chills, shock, aches and pains all over the body, nausea, and vomiting is common and can occur acutely during each infusion. Thrombophlebitis of the injected vein is also common.

5.2.3 Liposomal Drug Delivery System

All the therapeutic options described above are far from ideal because of prolonged courses of treatment, large-scale treatment failures (except with amphotericin B), and serious toxicities. Fatal toxicities due to Sb^v, pentamidine, and amphotericin B may be as high as 7–10%. The search for a better therapeutic option has led to the development of a targeted drug delivery system in the form of liposomal preparations: the "magic bullet" approach.

The "magic bullet" concept is based on the premise that the selective delivery of drugs to the tissues where they are to exert their pharmacological effects will not only enhance the desired therapeutic result but also minimize the occurrence of unrelated responses or toxic side effects. To achieve this, researchers have employed a variety of strategies, including drug design (e.g., prodrugs), carrier molecules (e.g., antibodies), and the incorporation of drugs into macromolecules (e.g., liposomes). Vesicular, cellular, and particulate carriers are removed from the blood rapidly and almost exclusively by the reticuloendothelial system, in particular macrophages of

the liver and spleen. This makes VL an ideal disease for passive drug targeting. Liposomes are microscopic vesicles 20–100 nm in diameter that have the ability to carry the encapsulated drugs to specific sites. The intraphagocytic nature of the parasite renders it highly susceptible to targeted drug delivery using liposomes, as the drug-laden liposomes are selectively taken up by the reticuloendothelial cells, especially in the liver and spleen, where they end up in the lysosomal apparatus. Here they are disrupted, and the drug is released, acting either locally or after diffusion outside the organelle in other cell compartments. As the drug is targeted to those very cells which host the parasite, it may be more effective than conventional amphotericin B. Moreover, there is striking decrement in systemic toxicity, as only minimal quantities of the drug are released in the free form.

Liposomal amphotericin B (AmBisome; Gilead Sciences, Foster City, CA, USA) is the most commonly used drug worldwide. In India, several studies have shown that small doses of AmBisome can cure a large number of patients. In a pilot study, 6 mg/kg of AmBisome cured 100% of 10 patients [9]. In another study in patients with refractory VL, AmBisome given in doses of 0.75, 1.5, and 3 mg/kg on five consecutive days cured 89, 93, and 97% of patients, respectively [10]. No significant adverse events were observed. In another study, a single dose of 5 mg cured 91% of patients [11]. Similarly, in a multicenter trial, 90% of 203 patients were cured with a single 7.5 mg/kg dose of AmBisome [12].

Unfortunately, due to the high cost of AmBisome (25–30 times the cost of conventional amphotericin B), even a highly attractive single-dose, low-dose regimen remains beyond the reach of most affected patients. Fortunately, the World Health Organization (WHO) has now negotiated a preferential price of US$20 – offering a 90% discount of the usual US$200 – for a 50 mg vial of AmBisome for use in developing countries. This cost reduction makes use of AmBisome now feasible throughout all the endemic regions of South Asia.

5.2.4 Paromomycin

Paromomycin (aminosidine), obtained from cultures of *Streptomyces rimosus*, belongs to the class of aminocyclitol-aminoglycosides and possesses not only antibacterial but additional antiprotozoal activity effective against *Leishmania*, *Entamoeba*, and *Cryptosporidium*. Aminoglycosides as a rule have poor intestinal absorption, and paromomycin is no exception. Therefore, parenteral preparations have been developed and used in VL. The general consensus is that a 21-day course of paromomycin base (11 mg/kg/day) is well tolerated. It has been strongly considered as a first-line treatment in Bihar. In the phase 3 trial in India in VL, the most common adverse event with paromomycin was injection site pain (55%). However, most of these cases were common toxicity criteria grade 1, and only one patient discontinued the drug due to injection site pain. None of the patients developed nephrotoxicity. Seven patients (2%) in the paromomycin group had ototoxicity

manifested as transient threshold shifts at high frequency, which returned to near baseline value during follow-up. An unexpected adverse event was a rise in hepatic transaminases in 31 patients (6%) [13]. The drug is now being produced in India, and an adult treatment course is expected to cost US$15.00. Its extremely affordable cost, coupled with a 3-week treatment schedule, makes it an attractive treatment option.

5.2.5 Miltefosine

A phase 1/2 dose escalation trial of miltefosine in India established that, in adults, a daily dose between 100 and 150 mg for 28 days is well tolerated and will cure most patients [14]. This was followed by a series of phase 2 studies confirming the results of the pilot study [15–17]. This led to a multicenter pivotal phase 3 study in which a high cure rate (94%) unquestionably established miltefosine as the first orally effective antileishmanial agent, thus revolutionizing antileishmanial therapy [18]. Its efficacy has also been reported in Sb^v-resistant cases. Depending on the individual weight, the recommended therapeutic regimen for patients weighing less than 25 kg is a single oral dose of 50 mg for 28 days, whereas individuals weighing more than 25 kg require a twice-daily dose of 50 mg for 28 days. The adverse effects of miltefosine included mild to moderate gastrointestinal disturbances that included vomiting and diarrhea in 40% and 15–20% of patients, respectively. However, at the end of the day, miltefosine has its limitations in that it induces gastrointestinal disturbances and renal toxicity. Nonetheless, these symptoms are reversible and are not a major cause for concern. As miltefosine is potentially teratogenic, it is contraindicated in pregnancy and in women of child-bearing age who do not use contraception.

5.3 Regulations and Policy

Irrational drug use is a potential threat to the lifespan of any drug, and this has probably contributed to the high level of treatment failure with Sb^v in Bihar, India [19]. The unrestricted availability of Sb^v in India has resulted in widespread misuse by unqualified practitioners, leading to incomplete treatment courses. According to a survey of drug resistance in India, only 26% of patients were treated according to WHO guidelines, and patients often stopped treatment on their own initiative [4]. The high reliance on the private sector and local pharmacists on the Indian subcontinent even today highlights the need for tighter regulation of the modalities of VL treatment, and for treatment to be made available at no cost [20–22]. When policymakers opt for combination therapy, they should take measures to limit the use of monotherapy, particularly where incomplete courses of treatment may result. The fact that miltefosine is available in India without prescription or regulation is worrisome, since this could facilitate the development of drug resistance [21].

5.4 Prevention of Drug Resistance

The problem of drug resistance in VL has been extensively reviewed elsewhere [23]. Treatment failure can manifest as initial treatment failure (failure to clear parasites at the end of the treatment course) or relapse (reappearance of parasites after initial cure, usually within 6 months of follow-up). Although Sb^v compounds have been successfully used throughout the world for decades, poor treatment response (mainly due to initial treatment failure) has increasingly been reported since the 1980s from Bihar, India, with geographical and temporal clustering in several hyperendemic districts [1, 24]. Although treatment outcomes could initially be improved with higher total doses, the improvement was only temporary [25–27]. In subsequent reports, therapy failed in up to 60% of patients that were newly diagnosed [2, 28]. At the same time, misuse of the drugs was reported [4]. Increased treatment failure has also been reported in Nepal, in districts that border Bihar [29]. Although treatment failure can be due to several causes – including factors related to drug, host, and parasite – substantial evidence exists that acquired drug resistance is a key issue. Reduced drug sensitivity has been reported with *L. donovani* strains from unresponsive cases in vitro [30, 31]. Reduced susceptibility to Sb^v has also been reported with *L. infantum* in both human beings and animals [32–34]. In these studies, post-treatment isolates had reduced sensitivity compared with pretreatment isolates, supporting the notion of acquired drug resistance. However, more recent studies have reported less clear associations of in vitro susceptibility and clinical outcomes, underscoring the need for improved and standardized methods [35]. Limited understanding of the mechanism of resistance toward Sb^v and the shortcomings of drug sensitivity assays make it difficult to predict the risk of acquired resistance in other regions or drugs, and to assess the need for combination therapy to help prevent resistance.

However, on the basis of the available evidence, acquired drug resistance should be considered a potentially serious threat to VL control, and comprehensive strategies should be developed, including the use of combination therapy [1, 3, 20, 23].

5.5 Rationale for Combination Chemotherapy

For individual drugs, the ease with which resistance develops will mainly depend on the parasite burden, the probability of spontaneous development of resistance mutations, and the fitness cost associated with those mutations [36]. The level and pattern of drug use in a population constitutes the selection force for the development of resistance, and intact host immunity is generally thought protective. The potency of the drug, therapeutic index, and pharmacokinetic properties of the drug also play a part [36]. Combination therapy can delay resistance if two drugs with different modes of action and mechanisms of resistance are used. The combination of synergistic drugs is preferred, because if more effective replication can be inhibited, resistance is less likely during treatment. For resistance prevention, both

drugs should ideally have similar pharmacokinetics. If parasites always confront both drugs, the probability of the emergence of double-resistant parasites would be expected to be extremely rare (i.e., the product of their individual per-parasite probabilities). A rapid elimination phase minimizes the duration of subtherapeutic drug concentrations that can provide an opportunity for amplification or selection of resistant parasites [36, 37]. In studies of malaria, the combination of one very active drug with a short half-life with a slower-acting drug with a longer half-life to clear the remaining parasites has been explored as a way to shorten treatment duration and improve treatment compliance [38]. However, recent studies have focused on the terminal elimination phase of the second drug, which can act as a selective filter for resistant malaria parasites [39, 40]. Finally, drugs can be combined to target different biological stages of the infectious agent. This has been done for tuberculosis and malaria, although the drugs are essentially targeted at preventing relapse and only indirectly prevent or delay resistance.

5.6 Pharmacological Considerations for Combination Therapy

Although the mechanisms of action and resistance remain poorly understood for all antileishmanial drugs currently in use (except amphotericin B), they are all thought to act on different targets [41]. Recent findings from India suggest that field isolates from areas with high-level resistance to Sb^v show reduced sensitivity toward other antileishmanial drugs such as amphotericin B and miltefosine [42]. However, true cross-resistance between the various drugs has not been reported so far. Several combinations have shown activity enhancement in animal experiments [43]. Clear differences in pharmacokinetics exist. Miltefosine might be particularly vulnerable to the emergence of resistance, because of its narrow therapeutic index and long half-life, which has been estimated at around 7 days [44, 45]. Recent data from patients with cutaneous leishmaniasis suggest a terminal half-life of 31 days, with miltefosine still detectable 5–6 months after the end of treatment [46]. Resistant strains might be selected and amplified during this period because of subtherapeutic drug concentrations, either from relapsing patients, or from newly acquired infections [44]. If confirmed, this might have important repercussions for the risk of emerging resistance and the duration of contraceptive measures. Paromomycin has a short half-life (2–3 h in patients without VL), but has a low therapeutic index. Resistance can easily be induced in vitro [47], and clinical resistance has been noted with its antibacterial use [48, 49]. Most of a Sb^v compound (about 99%) is eliminated within a few hours, followed by a slower elimination phase with a half-life of 76 h [50]. At least in East Africa, these drugs remain highly effective. Amphotericin B could be thought less likely to induce resistance, given its high efficacy and mechanism of action [19, 23, 51, 52]. Although resistance can been induced in vitro, clinical cases of amphotericin-B resistance are rare. Liposomal amphotericin B has a bioavailability in tissues for several weeks despite a relatively shorter plasma half-life [53, 54]. Given this long tissue half-life, a single dose of liposomal amphotericin B followed by daily administration of a second drug (e.g., sodium stibogluconate,

paromomycin, or miltefosine) would result in simultaneous exposure of the parasite to both drugs. The use of a single dose of liposomal amphotericin B (10 mg/kg) in monotherapy has proven to be extremely effective in India [55]. This simple and effective approach completely resolves the issue of compliance.

5.7 Compliance with Treatment

Besides the intrinsic characteristics of the combination regimen, compliance with the regimen also affects the risk of drug resistance. All conventional monotherapies (apart from liposomal amphotericin B) require a long treatment duration (21–30 days), making compliance more challenging. This is of particular concern for treatment with miltefosine, the only oral drug, for which the risk of premature treatment interruption is high. Even in a phase 4 trial, 4.5% of patients were lost to follow-up before the end of treatment, and 14.5% were not available for assessment by 6 months after treatment [56]. Thus, except in the case of high-dose single-injection AmBisome, monotherapy should be completely eliminated, including in the private sector. The shorter treatment duration might help to increase compliance, as has been the case for patients on combination regimens for malaria [3]. The lower costs to patients associated with shortened combination therapy could also improve access to and acceptance of VL treatment. Directly observed treatment strategy, which has been successfully used for tuberculosis, must be employed to ensure good compliance with treatment, although this will increase the indirect and direct costs marginally [57]. The elimination program for VL in South Asia has opted for miltefosine as a first-line drug, but the long treatment regimen, domiciliary treatment, and adverse events are strong deterrents to good compliance. It will be necessary to engage in the monitoring of clinical treatment outcomes and pharmacovigilance to ensure effective management of the VL elimination program [57].

5.8 Response to Treatment in AIDS-Related Kala-Azar

Treatment results in T-cell- and cytokine-deficient animals suggest that CD4 T-cell-depleted patients who have VL associated with acquired immune deficiency syndrome (AIDS) would respond poorly to Sb^v but satisfactorily to amphotericin B, and that they would also be likely to relapse if the initial treatment successfully induced an apparent clinical response and the drug was then discontinued. Taken together (but with variability in treatment regimens and definitions of efficacy), most reports from southern Europe, where coinfection has been best demonstrated [58], appear to confirm the following: (a) overall, approximately 50% of patients fail to respond initially to Sb^v in a region where 0–5% of otherwise healthy individuals are Sb^v unresponsive [59]; (b) of a total of approximately 50 coinfected patients treated with some form of amphotericin B, 90% showed initial responses; and (c) relapse rates in HIV-related kala-azar after any treatment is discontinued are 50%

and up to 90–100% [60]. Results from Spain, however, in the only randomized controlled study in HIV-associated kala-azar [6], provided a different finding in that the initial efficacies of both Sb^v (66% response) and amphotericin B (62% response) were reduced. Since this study did not include secondary prophylaxis, the majority of initial responders to either treatment relapsed. However, while once-monthly injections of amphotericin B lipid complex or AmBisome may prevent symptomatic recurrences [61, 62], no consensus has been reached about what constitutes optimal maintenance treatment in such patients.

5.9 Conclusion

In addition to intrinsic pharmacologic features, there are a number of human parameters that may favor the emergence and spread of leishmanial resistance. These include poor compliance, expensive treatment, availability of antileishmanial drugs over the counter, and limited access to healthcare facilities for early diagnosis and treatment. Given the current situation of the widespread emergence of antimonial resistance in India, there is growing concern about preserving the efficacy of novel antileishmanials. Such a strategy should focus on the following approaches:

(1) Treatment of VL should be based on guidelines for prompt diagnosis, selection of first-line drugs, management of cases unresponsive to antimonials, and HIV-coinfected cases;
(2) In order to enhance compliance, directly observed therapy for antileishmanials should be implemented, as in tuberculosis control programs;
(3) VL cases should be treated early in order to avoid further transmission of resistant parasites in the community;
(4) Distribution of and clinical response to antileishmanials should be monitored;
(5) Antileishmanial treatment should be provided free of charge through the healthcare system;
(6) The emergence and spread of antileishmanial resistance should be monitored; and
(7) The efficacy and safety of combination regimens should be evaluated in large trials, and if successful, they should be immediately implemented.

Acknowledgment This work was supported by NIAID, NIH TMRC, Grant No. I-P50AI074321.

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Chapter 6
Combination Therapy for Leishmaniases

Farrokh Modabber

Abstract After more than 50 years of using antimonials for the treatment of visceral leishmaniasis (VL), a number of drugs have recently become available, including miltefosine, paromomycin, and amphotericin B deoxycholate (Ampho B) plus the formulations of liposomal Ampho B (LamB). Miltefosine requires 28 days of treatment, is potentially teratogenic, and causes gastrointestinal disturbances. Because of its high cost and side effects, and because patients feel relief from VL symptoms after only a few days of treatment, full compliance is low and the probability of increased resistance is high. Paromomycin is inexpensive, but mild or moderate injection pain can result in low compliance and thereby increase the probability of resistance. Ampho B is effective if tolerated by patients but requires 15 intravenous infusions during 30 days of hospitalization. LamB is highly effective with minimal side effects and can cure more than 95% of VL patients with a single infusion, but it is very expensive. Hence, the Drugs for Neglected Diseases initiative (Geneva, Switzerland) in collaboration with the Indian Medical Research Council (Rajendra Memorial Research Institute, Patna, India) and the Kala-Azar Medical Research Center (Muzaffarpur, India) initiated studies to assess the safety and efficacy of short-course two-drug combination therapies for the treatment of VL in India. Results are better than expected, with high tolerability and higher efficacy than seen to date with any monotherapy.

Keywords Combination therapy · Immunochemotherapy · Immunotherapy · Leishmaniasis vaccine · Leishmaniasis · Prophylaxis

Abbreviations

Ampho B	Amphotericin B deoxycholate
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BCG	bacillus Calmette-Guerin

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_6,

DOT	Directly observed treatment
HIV	Human immunodeficiency virus
LamB	Liposomal amphotericin B
PKDL	Post-kala-azar dermal leishmaniasis
RMRI	Rajendra Memorial Research Institute
VL	Visceral leishmaniasis
VLEP	Visceral leishmaniasis elimination program
WHO	World Health Organization

6.1 Introduction

Because of increased parasite resistance to antimonials – the first-line drug for visceral leishmaniasis (VL) in Bihar, India [1] – it became necessary to change the policy for treatment. Ampho B replaced antimonials in Bihar, but due to its potential toxicity, the required 1-month hospitalization, and the 15 intravenous infusions, Ampho B is not practical for the VL elimination program (VLEP) in the Indian subcontinent. Subsequently, when miltefosine, the first oral drug for VL, was developed, VLEP adopted miltefosine [2]. However, because of its potential teratogenicity for humans, miltefosine cannot be used in pregnant women and in women of child-bearing age without a pregnancy test and contraception for at least 3 months. In addition, miltefosine requires 28 days of treatment. Compliance was shown to be low due to the long duration of treatment, cost of the drug, and side effects, especially gastrointestinal disturbances. Given the ease of developing resistant parasites in vitro [3], it is expected that miltefosine monotherapy, particularly without a directly observed treatment (DOT) program, would soon lead to an increase in resistant parasites.

Paromomycin, an old drug in the class of aminosidines, was developed in injectable form and registered in India in 2006 [4]. Although inexpensive and relatively safe, paromomycin requires 21 daily injections that can be painful. Also, based on laboratory evidence, paromomycin could become ineffective for the treatment of VL if used as monotherapy due to an increase in resistant parasites [5].

Although the efficacy of LamB against VL was demonstrated in the 1990s [6], and its safety established through its wide use in fungal infections, this drug has been inaccessible to patients because of its extremely high cost. A reduced cost negotiated by the World Health Organization (WHO) has made it possible for LamB to be considered for use in VL-endemic countries. Although the cost is still high, its efficacy and lack of toxicity (compared to Ampho B) make it a very attractive drug for the treatment of VL, at least in India, where a single dose has been shown to cure about 96% of patients [7]. So far, no infective *Leishmania* resistant to Ampho B has been shown either in real life or in a laboratory setting [8] but its use in leishmaniasis has been very limited. Therefore, it is impossible to predict how long it will take for resistant parasites to emerge. In order to reduce the probability of development of drug-resistant *Leishmania* and to reduce the duration of treatment – and thereby also the toxicity and side effects of these drugs when used as monotherapy – the Drugs

for Neglected Diseases initiative sponsored a clinical trial and assessed the safety and efficacy of combinations of available drugs when given over shorter periods.

6.2 Drugs Currently in Use

6.2.1 Pentavalent Antimonials

Two pentavalent antimonials are currently available: meglumine antimoniate and sodium stibogluconate. They are chemically similar, and their toxicity and efficacy in VL are related to their active ingredient, pentavalent antimony (Sb^{5+}). Meglumine antimoniate solution contains about 8.1% Sb^{5+} (81 mg/mL), whereas sodium stibogluconate solution contains about 10% Sb^{5+} (100 mg/mL). In general, children tolerate antimonials better than adults. Common side effects are anorexia, vomiting, nausea, malaise, myalgia, headache, and lethargy. Electrocardiographic changes depend on dosage and duration of treatment (cardiotoxicity is dose-dependent and usually occurs after 2 weeks of treatment), the most common being T-wave inversion, prolonged Q-T interval, and, rarely, arrhythmia. Nephrotoxicity is a rarely reported side effect.

Initial treatment of a parasitologically proven case of VL should be based on a daily injection of 20 mg of Sb^{5+} per kg of body weight. Injections are normally given for 4 weeks. The duration of treatment varies from one endemic area to another, but should be individually determined for each country and each endemic focus. Treatment should be given under the supervision of medical personnel. Treatment with antimonials is usually well tolerated, but if serious side effects arise (in most cases related to hepatotoxicity and cardiotoxicity), it should be interrupted temporarily. Rescue treatment is advised if other drugs are available. If relapses occur, patients should be treated with other available anti-leishmaniasis drugs if possible.

6.2.2 Ampho B

Ampho B was formerly used as a second-line treatment for patients not responding to antimonials. However, in recent years it has been recommended as first-line treatment in Bihar, India, due to parasite resistance to antimonials. The recommended dose is 15 mg⁄kg given at 1 mg⁄kg as a slow (4–6 h) infusion every other day for 30 days. Adverse events, primarily hepatotoxicity, may occur in 5–7% of patients. Relapse or lack of response occurs in 1–2% of cases. Treatment should always be given in hospital because of the risk of nephrotoxicity and cardiotoxicity.

6.2.3 LamB

The toxicity of Ampho B is greatly reduced when it is given in association with lipids. Several different formulations exist, but LamB is the most frequently used (Fig. 6.1). Doses of up to 21 mg⁄kg over 10 days have been given with over 98%

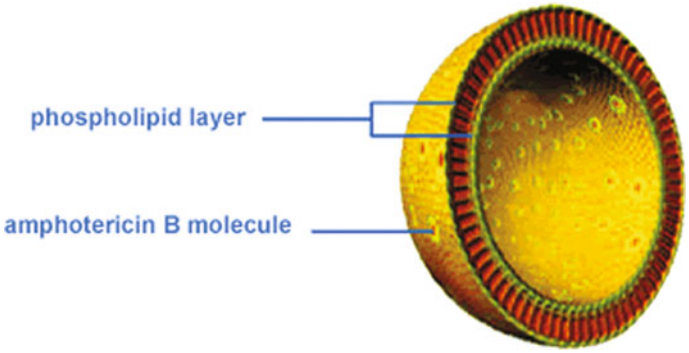

Fig. 6.1 Schematic presentation of LamB. From the package insert: http://www.fujisawa.com/medinfo/pi/pi_page_amb.htm

efficacy. However, a single dose of 10 mg⁄kg has been reported to cure about 95% of patients in a trial in India [7]. Patients in Africa and Latin America respond differently than those in India and may require higher doses of LamB. The guideline for LamB treatment of VL in Sudan is 3 mg⁄kg on alternate days for 14 days (total 21 mg⁄kg) and that for Kenya is 14 mg⁄kg total given in seven daily infusions of 2 mg⁄kg per day. For VL in Latin America caused by *L. chagasi* (*L. infantum*), the total suggested dose is 20 mg⁄kg administered over 5–7 days. However, further studies are required to find the most cost-effective dose.

6.2.4 Paromomycin

Produced from culture filtrates of *Streptomyces krestomyceticus* and active against various micro-organisms, the aminosidine paromomycin has been registered in India for the treatment of VL but is not yet widely available. Daily intramuscular injections of 11 mg⁄kg of paromomycin (the equivalent of 15 mg of paromomycin sulphate) given for 3 weeks have been efficacious in India and show a safety profile similar to that of other drugs of its class. The most commonly reported adverse drug reactions are injection site pain, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) value elevations (above three times the upper limit of the normal range), pyrexia, and an abnormal audiogram. These effects are usually mild to moderate and transient or reversible after the end of treatment [9].

In Sudan, the efficacy of paromomycin at the same dose used in India was less than 50%. In a dose-response study in Sudan to define the required dose, an increased dose of 15 mg of paromomycin (equivalent to 20 mg of paromomycin sulphate) resulted in 80% efficacy [10]. The advantages of paromomycin are its low cost and high stability. However, continued use of paromomycin as a monotherapy will eventually make this drug useless because of the development of drug resistance, as seen with other aminoglycosides and as mentioned above [5]. No data exist on paromomycin efficacy in Latin America.

6.2.5 *Miltefosine*

Miltefosine (hexadecylphosphocholine) is the first oral drug active against VL and is available as 10 mg or 50 mg capsules. The recommended dose for children over 2 years of age and adults is 1.5–2.5 mg⁄kg (maximum total dosage 100 mg per day). Miltefosine efficacy against VL is high in the Indian subcontinent [11], but being a potentially teratogenic drug, it cannot be given to pregnant women, and contraception must be used by women of child-bearing age. Compliance is a concern, since the drug has some gastrointestinal toxicity and patients usually feel relief of VL symptoms after a few days of treatment. Other common side effects include: vomiting, diarrhea, and elevation of liver enzymes and serum creatinine. These effects are usually mild to moderate and transient or reversible at the end of treatment. Less common side effects include thrombocytopenia, anorexia, and abdominal pain. Data are limited from other disease foci in Africa and Latin America. One phase 3 study in Ethiopia suggested a similar safety and efficacy profile to that of sodium stibogluconate in patients not infected with the human immunodeficiency virus (HIV) [12].

6.3 Combination Therapies

Monotherapy with available drugs is of long duration: 21 days for paromomycin, 28 days for miltefosine or antimonials, and 30 days for Ampho B. LamB treatment is short (1–10 days depending on the country). Toxicity and side effects usually increase with time during treatment. Considering the side effects, the cost of treatment, and the fact that patients feel better after a few days of taking the medications (particularly miltefosine), compliance can be low without a DOT program. Low compliance can increase the probability of developing resistance. The cost of LamB and miltefosine are still high despite WHO negotiations with manufacturers to reduce it. Therefore, combination of lower doses of these drugs with paromomycin (the most affordable anti-leishmanial drug) would reduce the cost of treatment. Combination treatments have the advantage of:

- Shortening duration of treatment compared with available monotherapies, thereby reducing side effects and cost to patients (except in the case of paromomycin as a monotherapy) and increasing compliance;
- Reducing the overall dose of drugs, thereby reducing toxic effects;
- Reducing the probability of developing resistant parasites, thereby prolonging the effective life of the available drugs.

Several combination treatment trials had favorable results (for a recent review see van Griensven et al[13]). A combination of paromomycin and sodium stibogluconate increased the cure rate in VL patients in Bihar, India, compared with sodium stibogluconate alone (non-responsiveness of 40–60% [14]). The combination of a single dose of 5 mg⁄kg of LamB and various doses of miltefosine produced over

95% efficacy in a phase 2 trial with 40 patients per arm [15]. A definitive, randomized, open-label, hospital-based, non-inferiority ($\Delta > 7$) trial will soon be completed for comparing the standard treatment using Ampho B (1 mg⁄kg every other day for 30 days) with combination treatments using two drugs: a single dose of LamB (5 mg⁄kg) with 7 days of miltefosine (instead of 28 days); or a single dose of LamB (5 mg⁄kg) with paromomycin for 10 days (instead of 21 days); or a combination of miltefosine and paromomycin for 10 days instead of 21–28 days (Fig. 6.2). Six hundred thirty-four parasitologically confirmed VL patients from two centers in Bihar (Patna and Muzaffarpur) were enrolled, treated, and followed up for 6 months. All three combinations showed a safety profile better than that of the standard Ampho B (Sundar et al., personal communication). In the Ampho B group, adverse reactions included elevated liver enzymes or serum creatinine. There was one fatal cardiac infarct (a known but rare reaction to Ampho B) in a high-risk 59-year-old male patient. In the combination groups, only one patient showed an immediate hypersensitivity reaction to the test injection of LamB. Laboratory parameters measured during and after treatment (hematology, biochemistry) showed no clinically significant variations in the combination groups. Laboratory parameters (hematology and biochemistry) during the treatment period and up to day 45 follow-up showed no significant differences between the three combination groups, but they all showed significantly lower increases of creatinine, AST, ALT, and blood urea nitrogen, and a significantly better recovery from anemia than under the standard Ampho B

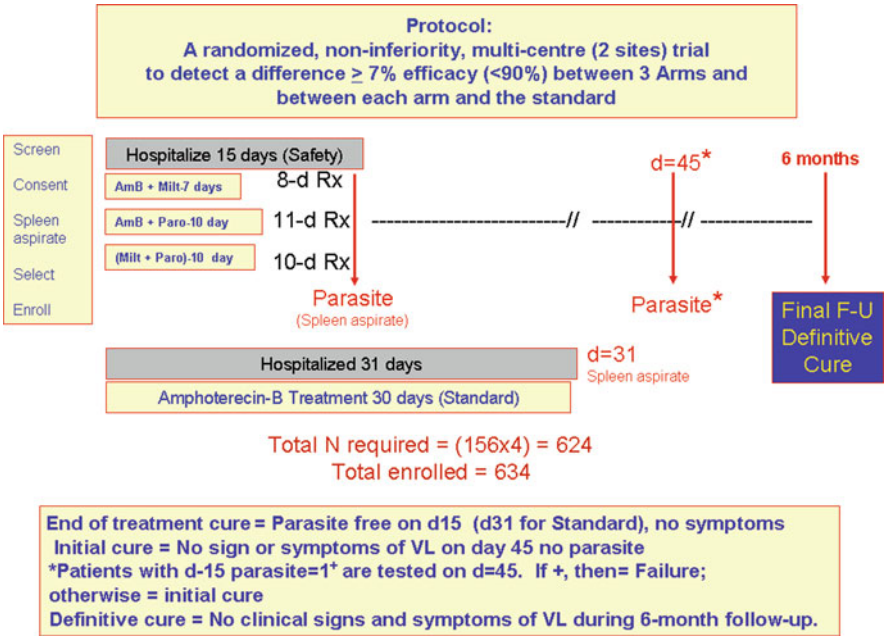

Fig. 6.2 Description of the trial protocol

treatment. Based on the safety profile, and if these results can be confirmed in field conditions, it may be possible to forgo monitoring patients undergoing combination therapy. If this is confirmed, a very short hospitalization (1–2 days) followed by outpatient treatment will make the combination treatment more cost-effective than the other modalities of treatment presently available. However, further studies with larger patient populations under field conditions are needed to support this finding.

6.4 Future Trends for the Treatment of Leishmaniases

A strong immunity is usually developed after recovery from most forms of leishmaniasis in immunocompetent individuals. In immunocompromised hosts, the disease returns when chemotherapy is stopped. Hence, maintenance treatment is required (Fig. 6.3a). In immunocompetent hosts, a protective immune response develops following chemotherapy that keeps the parasite under control (Fig. 6.3b). Indeed, it is generally believed that sterile immunity does not exist in leishmaniasis, and that the parasites are controlled by the host's effector immune response. This theory is supported by the frequent development of fulminating leishmaniasis in patients who have recovered from a VL infection but subsequently become infected with HIV or are otherwise immunocompromised by immunosuppressive drugs used for cancer therapy. Therefore, the protective immune response is an important part of recovery from leishmaniasis. The goal of immunochemotherapy is to use a vaccine

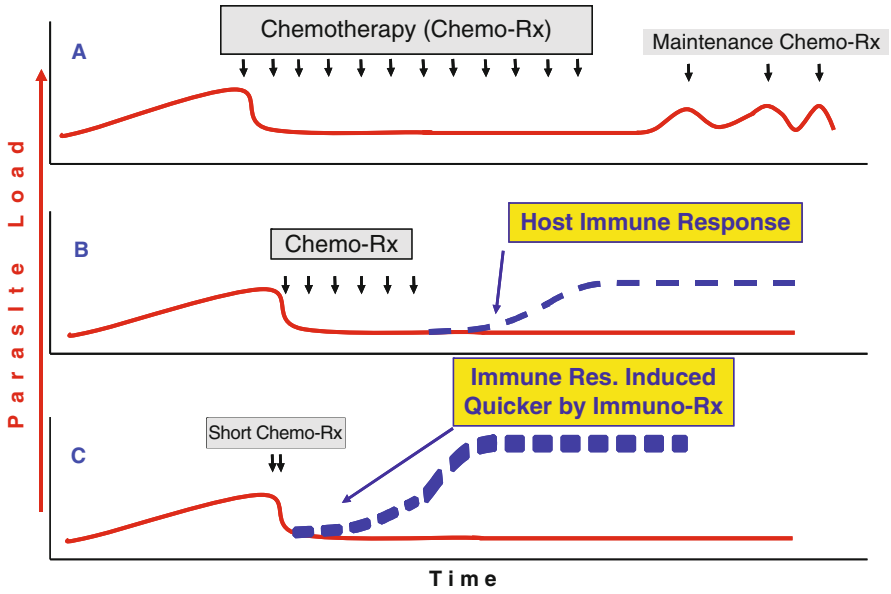

Fig. 6.3 Conceptual representation of chemotherapy plus immunotherapy in different forms of leishmaniasis (from [20])

or immunomodulators to induce the protective response rapidly to cure the disease (Fig. 6.3c). The concept of immunostimulation during chemotherapy is not new and not specific to leishmaniasis. Immunomodulators have been used for cancer therapy and new ones are under development.

Immunotherapy with or without chemotherapy has been used for treatment of cutaneous leishmaniasis. In Venezuela, killed *L. mexicana* plus live bacillus Calmette-Guerin (BCG) was used instead of antimonials to reduce cost and side effects [16]. In Brazil, to reduce the dose of antimonials to minimize toxicity and cost, daily doses of killed *L. amazonensis* vaccine (Mayrink's vaccine) were administered together with a low dose of antimonials (8 mg⁄kg per day) and cured 100% of patients compared to 4% after four cycles of treatment using low-dose antimonials alone [17]. One treatment cycle consists of 10 daily injections followed by 10 days of rest. The vaccine was registered in Brazil as an adjunct for low-dose chemotherapy. There have been several case studies of immunochemotherapy for mucosal leishmaniasis using first-generation vaccines or recombinant antigens of *Leishmania* plus granulocyte-macrophage colony-stimulating factor and antimonials [18]. However, more studies of this approach are needed.

An exploratory, hospital-based, randomized, controlled trial in Sudan [19] on persistent cases of post-kala-azar dermal leishmaniasis (PKDL) – involving lesions lasting longer than 6 months and difficult to cure with drugs alone – showed that the cure rate using immunochemotherapy was significantly better than with chemotherapy alone (final cure rate at day 90 was 100% versus 40%; $p < 0.004$). The vaccine was a mixture of killed *L. major* adsorbed to alum plus BCG, given four times at weekly intervals. The exact mechanism involved in enhancing the cure rate is not fully understood. However, in PKDL lesions there is an increase of αβ T-cells but a reduction of CD1 antigen-presenting cells. Even in the presence of effector Th-1 cells, parasites are not killed and lesions persist. External interferon-γ does not cure all patients with leishmaniasis. This may be due to down-regulation of B7-1 and up-regulation of B7-2 by interleukin-10, thereby leading to a predominant Th-2 response, which can be diverted by immunomodulation using a vaccine or possibly other immunomodulators [19]. It should be noted that a minority of PKDL patients attained cure without showing a pure Th-1 response. Therefore, the classical Th-1/Th-2 dichotomy described in mice does not necessarily apply to humans. Further studies are needed. However, since PKDL is believed to be a reservoir of infection and lesions may last for years without treatment, treatment should be given. Patients with PKDL, which may develop months or years after VL but in some cases develops concurrently, generally feel healthy and do not seek treatment, which is long and expensive (2–4 months). An affordable short-course treatment of PKDL is urgently needed for the elimination of VL, and immunochemotherapy seems to be a possible approach for reducing duration of treatment.

Several new immunomodulators are being developed for cancer therapy; with adequate knowledge of their mechanisms of action, selected agents should be tested for the treatment of VL and other forms of leishmaniasis.

6.5 Recommendation

To protect currently available drugs against the development of parasite resistance and to reduce both treatment time and cost, thereby increase compliance, monotherapy with antimonials, paromomycin, and miltefosine should be replaced by combination treatment in India. Once data from India are confirmed in Bangladesh and Nepal, combination treatment should be adopted throughout the Indian subcontinent. The choice of treatments to be used in combination depends on the possibility of administering pregnancy tests and ensuring contraceptive use in women of child-bearing age for miltefosine, and the availability of an infrastructure for the proper administration of LamB. At present, the combination of miltefosine and paromomycin is the most cost-effective treatment (Meheus et al., personal communication). Single dose LamB is a powerful tool for control of VL in Indian subcontinent and has been recommended recently by WHO for near-term implementation due to low toxicity and being a single dose.

Acknowledgment The editorial assistance of Federica Giovannini was highly appreciated.

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Part III
Diagnostic Strategy Enhancing Kala-Azar Elimination Program

Chapter 7
Challenges in the Diagnosis of Visceral Leishmaniasis on the Indian Subcontinent

Suman Rijal, Francois Chappuis, and Marleen Boelaert

Abstract Visceral leishmaniasis (VL) or kala-azar, an endemic vector-borne disease, affects the populations of the lowest socio-economic strata on the Indian subcontinent – a group that has limited access to proper health care. Untreated, kala-azar is almost always fatal, and the drugs currently in use are toxic and/or expensive. Thus, confirmation of diagnosis before starting therapy is crucial. Early diagnosis and treatment are a key strategy of the recently launched kala-azar elimination program in Bangladesh, India, and Nepal. To achieve early diagnosis, VL care must be decentralized to primary health centers, which has become possible since the advent of rapid diagnostic tests. Parasitological diagnosis is limited to referral hospitals and specialized VL treatment centers. Two serological tests for field use – the direct agglutination test and the rK39 immunochromatographic test – have both shown excellent performance on the Indian subcontinent, but the latter is preferred, as it is simpler to use. The proper implementation of these diagnostic strategies within the VL elimination program involves not only the procurement, training, and supervision of staff, but also quality control both before and after deployment in the field. The logistical requirements are enormous, and therefore standardized guidelines for procurement and quality control must be established.

Keywords Kala-azar · Visceral leishmaniasis · Kala-azar elimination program · Diagnostic test · Rapid diagnostic test · Access to health care · rK39 immunochromatographic test · Direct agglutination test

Abbreviations

CI	Confidence interval
DAT	Direct agglutination test
FGT	Formol gel test
ICT	Immunochromatographic test

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_7, © Springer Science+Business Media B.V. 2011

KAtex	Latex agglutination test
PCR	Polymerase chain reaction
RDT	Rapid diagnostic test
VL	Visceral leishmaniasis

7.1 Introduction

Visceral leishmaniasis (VL) or kala-azar is a major public health problem in Bangladesh, India, and Nepal, and the populations affected represent the poorest of the poor living in remote rural regions [1]. Over 60% of the annual VL cases worldwide are from this region, and 150 million people are estimated to be at risk [2]. In addition, the endemic regions within these three countries constitute a contiguous area. The causative agent is *Leishmania donovani*, and studies of the genome of parasites from these countries have shown a very homogeneous population regardless of geographical origin of the samples [3].

Kala-azar is fatal if untreated, and even with treatment up to 5% may die, as some VL drugs are toxic [4]. Increased fatality has also been found to be associated with advanced disease [5]. Increasing treatment failure of the anti-VL drug sodium stibogluconate on the Indian subcontinent (in particular, India and Nepal) has been well documented over the last decade [6, 7]. A recent study from Nepal showed a significant association of treatment failure with delayed diagnosis [8]. Therefore, there is a need for a diagnostic test that is not only highly accurate but also can easily be used at the peripheral health facilities where the majority of kala-azar patients are seen.

In the kala-azar elimination program jointly launched in Bangladesh, India, and Nepal in 2005, early diagnosis and appropriate treatment are key strategies [9]. Decentralization to the peripheral level health facilities – e.g., primary health care centers – will make VL care more accessible. The availability of a rapid diagnostic test (RDT), the rK39 immunochromatographic test (ICT) [10], and an oral drug, miltefosine, would make it feasible to implement this strategy [11].

This chapter will present a review of the currently available diagnostic tools for VL and their utility, current limitations to access to VL care, and issues in the implementation of the RDT in the kala-azar elimination program on the Indian subcontinent.

7.2 Diagnostic Tests for VL

Accurate diagnosis is crucial before starting anti-VL drugs. The clinical manifestations of kala-azar (fever, anemia, splenomegaly) are nonspecific and are also commonly seen in other diseases that occur in this region. In a phase 3 validation study for the diagnosis of VL in Nepal [12], the common differential diagnosis of VL included malaria, disseminated tuberculosis, hematological malignancies, and sepsis [13]. Applying the diagnostic test in suspected VL cases (fever of $\geq$2 weeks with palpable spleen), the proportion of VL cases ranged from 50% at a district

level hospital to 70% at a referral hospital [14]. Thus, a significant proportion of suspected VL cases at a peripheral level hospital would require referral to a higher level health facility for confirmation of the diagnosis, but most of these patients are treated empirically, as they cannot afford to visit these specialized hospitals.

The currently available diagnostic tests for VL can be broadly divided into nonspecific VL tests, parasite demonstration, serological tests, and antigen detection tests.

7.2.1 Nonspecific Tests

Nonspecific tests for VL include the demonstration of pancytopenia and the aldehyde or formol gel test (FGT). The former was found in a study of suspected cases in Nepal to have excellent specificity, but the sensitivity was only 16% [15]. The FGT, developed by Napier in the 1920s, is based on the demonstration of the increased gamma globulin levels found in kala-azar patients [16]. Due to the simplicity of this test, the non-feasibility of performing microscopy on tissue aspirates, and the absence of an easily administered alternative, the FGT remained in use in health facilities on the Indian subcontinent until only a few years ago. However, its sensitivity has been shown to be very low (35%), though the specificity is excellent [15].

7.2.2 Parasite Detection

Parasite detection can be broadly described as microscopy of tissue aspirates to demonstrate the presence of *Leishmania* amastigotes, along with culture and molecular biology techniques. Since the discovery of the parasite by Leishman and Donovan in 1903, microscopy has been considered the gold standard for confirmation of the diagnosis [17]. The sensitivity of microscopy depends on the tissue aspirate; for example, it is higher for spleen tissue (93–99%) than for bone marrow (53–86%). Splenic aspiration carries a 0.1% risk of life-threatening hemorrhage even in centers specializing in kala-azar. This procedure is therefore not suitable to perform at peripheral centers, as it requires technical expertise to perform and interpret, and facilities for blood transfusion and surgery must be available. Molecular biology tools such as polymerase chain reaction (PCR) have demonstrated excellent sensitivity even using blood samples, but the specificity was as low as 62% (95% confidence interval [CI], 51–72) in a phase 3 diagnostic study from Nepal [18]. PCR also requires sophisticated tools, limiting its use to research centers and university hospitals in the endemic countries [19], though efforts have been made to simplify the procedure by using less sophisticated technology [20].

7.2.3 Serological Tests

There are many test formats that detect antibodies to *Leishmania* from patients' serum, but most are not suitable for use in field conditions on the Indian

subcontinent. Tests based on indirect fluorescence antibody, enzyme linked immunosorbent assay, and Western blot techniques show high diagnostic accuracy but are considered too complicated for field use. The direct agglutination test (DAT) and the rK39 ICT are the two tests that are considered appropriate for field use; both have been extensively validated on the Indian subcontinent.

The DAT, a semi-quantitative test, requires microtiter plates in which increasing dilutions of a patient's serum or blood is mixed with stained, killed promastigotes of *L. donovani* [21]. The results are read with the naked eye after an overnight incubation. A recent meta-analysis that included 30 studies showed sensitivity and specificity estimates of 94.8% (95% CI, 92.7–96.4) and 97.1% (95% CI, 93.9–98.7), respectively [14]. The performance of the DAT was quite similar in the different endemic foci of India, East Africa, and Brazil. Though the DAT is simpler to perform than many other serological test formats as IFAT or ELISA, it still requires equipment (microplates, micropipettes), extensive training of laboratory technicians, and regular quality control; it is also relatively expensive (2–4 USD per test). DAT initially required transportation through a cold chain but the latter obstacle has been overcome by the development of a freeze-dried version available from two academic institutes in Europe that delivers a performance comparable to that of the liquid antigen [22]. A major drawback of the DAT is moreover that it is not commercially available and a number of academic institutes produce their "in-house" DAT antigen without standardisation between them. For all the above reasons and though the DAT has been extensively validated in most endemic areas, a consensus has grown that it is not the most appropriate technology for use in primary care centres.

The rK39 dipstick contains a 39-amino acid repeat that is part of a kinesin-related protein of *L. chagasi*. The repeat is conserved within the *L. donovani* complex. This test developed in an immunochromatographic format (dipstick) is suitable for field use, as it is easy to administer, and results – which are reproducible – can be obtained in 10–15 min. A meta-analysis that included 13 validation studies of the rK39 dipstick showed sensitivity and specificity estimates of 93.9% (95% CI, 87.7–97.1) and 95.3% (95% CI, 88.8–98.1), respectively, on the Indian subcontinent, while the sensitivity was lower in East Africa [14]. Meanwhile, a recent multi-center study has confirmed the excellent diagnostic performance of the rK39 dipstick in India and Nepal [23]. It is quite clear that the rk39 ICT is currently the best available VL diagnostic tool for use in the field; it has been appropriately recommended as a diagnostic test for use in the kala-azar elimination initiative on the Indian subcontinent.

However, serological tests have major limitations when used for VL diagnosis. As specific antibodies continue to be detected up to several years after cure in a high proportion of patients [24, 25], the test is useless in patients who come back after treatment with a suspicion of VL relapse. In addition, healthy people living in endemic areas may carry anti-leishmanial antibodies because of past or active asymptomatic infections. The seroprevalence in the healthy population in low transmission areas varied from around 5–15% in Nepal [26], while in India it was above 30% in areas of high transmission [27]. Thus, it is important to emphasize that

antibody tests must always be used in combination with clinical case definitions for VL diagnosis. The kala-azar elimination program encourages an active case finding approach to enhance earlier detection of kala-azar cases. This both benefits the individual with kala-azar and decreases transmission in the community. As the validation studies have used a passive case detection strategy, there is still a need to assess whether the performance of the diagnostic test is comparable when it is employed using an active case detection strategy that assumes a shorter duration of the illness before diagnosis.

7.2.4 Antigen Detection Tests

In theory, antigen detection tests are more specific than serological tests, as they avoid cross-reactions and can distinguish active from past infections. A latex agglutination test (KAtex) detecting a heat-stable low molecular weight carbohydrate antigen in the urine of kala-azar patients showed promising initial results when evaluated in the diagnosis of kala-azar [28, 29]. Several studies conducted in East Africa and on the Indian subcontinent showed good specificity but low-to-moderate (48–87%) sensitivity [14, 30, 31]. As to be expected with an antigen detection test, KAtex turns negative in a very high proportion of patients (97–100%) during antileishmanial treatment [32]. Apart from its low sensitivity, there are two practical limitations with KAtex. First, the urine must be boiled to avoid false-positive reactions, and second, it is difficult to distinguish weak positive from negative results, which affects the test's reproducibility [13, 30]. A simplified and improved form of KAtex is currently under development.

7.3 Access to VL Care

The determinants of access to VL care include awareness of the disease, the inclination to seek health care, and the accessibility and acceptability of diagnostic and treatment facilities.

The national control programs that have been operating for many years in endemic countries include information, education and communication (IEC) activities. Therefore, one might expect that the majority of the population in endemic areas would be knowledgeable about the disease and its transmission. However, a recent study showed that knowledge of the disease remained low in some regions. Fever, the most common symptom, was identified by 32, 72, and 92% of the interviewees from Bangladesh, Nepal, and India, respectively. In India and Nepal, almost all the respondents were aware that kala-azar was a curable disease, while in Bangladesh only 64% were aware of this [33].

Numerous studies have shown that local unqualified village health care workers are the first-choice health care providers of many patients, because of their easy accessibility [33–35]. As a next step on the health seeking path, patients tend to prefer private providers, local pharmacists, or government health care facilities. A

study from Bangladesh found that patients paid a median of seven visits to different providers before starting VL treatment [34], and a similar situation has been found in Nepal [35, 36]. As a consequence, catastrophic expenditures are often incurred by the families of these kala-azar patients to pay for provider fees, tests, drugs and transport, despite the existence of a control program providing free VL care. Over the last few years, efforts have been made by the health authorities to improve access to care by decentralizing VL treatment. Since VL is a focal disease, strategies for active case detection in the so-called "hotspots" are also being explored by the elimination initiative, to decrease the time to diagnosis and also prevent unnecessary visits to different care providers.

7.4 Issues in the Use of the RDT in the Field

The issues to be addressed concerning the field use of the RDT within the control program include the procurement and supply of the test kits, training and monitoring of their use by health care workers, and quality control.

After the validation of the rK39 ICT, RDT was accepted as a diagnostic test and has been introduced in the control program up to the primary health center level in all three countries. This has encouraged many manufacturers to start producing RDTs, and currently many brands of RDT using the dipstick format are available. However, many of them have not yet been scientifically validated, and some contain antigens other than rK39.It has been shown that the performance of kits of the same type (e.g., rK39 RDT) may differ among brands [37]. This has created a difficult situation for the procurement units of the Ministries of Health, because standard guidelines for the registration of diagnostic tests similar to those regulating drug qualityhave not yet been established. A major factor in purchasing policies is cost, which may discourage the selection of the scientifically validated brands.

The introduction of a new diagnostic test in a control program requires training the health personnel involved, which is a challenging task. The large number of health workers poses logistical challenges. Training manuals should be adapted to the level of individual health workers. In addition, the frequent transfers of health workers – which is quite common in these countries – and changes in the format of the RDT (dipstick or cassette versions) produce major difficulties.

Quality control of both the products procured and their use in the field is essential. Not only the performance of the different brands needs to be checked after the product reach the country, but also that of different batches of the same brand. Most RDTs must be transported and stored within specified temperatures, avoiding extremes of heat. In these endemic countries on the Indian subcontinent, where both the temperature and humidity can be quite high, transportation under ideal conditions may not always be practically possible.

To avoid treating asymptomatic *Leishmania* infections, RDTs should be applied in combination with a strict clinical case definition, as many healthy people in the endemic region may also test positive for the disease [38, 39]. Currently in these

countries there is no set protocol for quality control after procurement of the RDT or monitoring its use in the field.

In 2009 WHO/TDR has set up an international laboratory network in endemic countries to perform quality assurance for existing VL RDTs. The evidence generated by this network will help the national control program in their purchasing policy for RDTs.

7.5 Conclusion

Diagnostic tests in the VL elimination program will only have an impact if they are widely available to patients. The currently available RDT (rK39 ICT), though not ideal, is essential for the success of the kala-azar elimination initiative. There is a need for an RDT that is more specific for acute stage VL disease, possibly based on antigen detection, to assess cure and relapse after VL treatment. Standards for the registration and quality control of RDTs must be established within each of the control programs.

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Chapter 8
The Potential of Urinary Tests in the Management of Kala-Azar

Eisei Noiri, Yoshifumi Hamasaki, Kousuke Negishi, Takeshi Sugaya, Kent Doi, Toshiro Fujita, Yukihisa Osada, Yoshitsugu Matsumoto, and Kazi M. Jamil

Abstract The diagnostic process of disease detection and the disease management after therapy has been initiated differ from one disease to the next. The process of diagnosing kala-azar has recently been simplified. The time-consuming pathological exam that was formerly needed has been replaced by a convenient blood test. But once treatment has begun, the monitoring of disease activity still relies on clinical findings and laboratory data in endemic areas. In this chapter, we propose the use of the urinary biomarker, fatty-acid-binding protein 1 (FABP1), for monitoring kala-azar disease activity and drug-induced side effects. The FABP1 assay, developed as an enzyme-linked immunosorbent assay, was developed in the form of an immunochromatography (dipstick) urine test in our project for use in areas with a high concentration of kala-azar infection. We expect that the FABP1 dipstick test will prove invaluable as a triage tool and for monitoring the severity of the disease. In addition, we discuss the potential usefulness of urinary interleukin-18, a biomarker for acute kidney injury, in monitoring kala-azar activity. Further studies of this and other recently discovered urinary biomarkers are needed.

Keywords Dipstick urine test · Fatty-acid-binding protein 1 · Interleukin-18 · Kala-azar · Leishmaniasis · Urinary biomarker

Abbreviations

AKI	Acute kidney injury
BUN	Blood urea nitrogen
CDDP	Cisplatinum
CKD	Chronic kidney disease
COX-2	Cyclooxygenase 2

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_8,

CPB	Cardiopulmonary bypass
EC	Endemic control
ELISA	Enzyme-linked immunosorbent assay
FABP	Fatty-acid-binding protein
FGS	Focal glomerulosclerosis
HNE	4-Hydroxy-2,3-Nonenal
IFN-γ	Interferon-gamma
IL	Interleukin
KIM-1	Kidney injury molecule-1
L-FABP	L-type fatty-acid-binding protein, FABP1 fatty-acid-binding protein 1
NAG	N-acetyl-β-D-glucosaminidase
NGAL	Neutrophil gelatinase-associated lipocalin
PCR	Polymerase chain reaction
PMX-F	Polymyxin B-immobilized fiber
ROC	Receiver-operating-characteristic
RT	Room temperature
SCr	Serum creatinine
SSG	Sodium stibogluconate
Th1, Th2	T helper cell types 1 and 2

8.1 Introduction

Formerly, the diagnosis of kala-azar required parasite detection in organ aspirates performed by specially-trained pathologists. Now a simpler process has been developed using a test for rK39 that aids in the diagnosis of kala-azar [1]. But while a new standard diagnostic procedure for kala-azar has been established, the monitoring procedure for disease activity after starting kala-azar therapy varies in the field based on clinical symptoms such as fever or the presence of splenomegaly. Blood tests such as those for erythrocyte sedimentation rate, complete blood count, and prothrombin time are too elaborate to be suitable for large-scale monitoring in the field. And the antibody-based immune-detection tools used for detecting kala-azar are not helpful in monitoring disease activity since the antibody-positive phase is sustained in the immune system for a long period after the onset of the disease [2]. In addition, low-tech monitoring systems are needed for use in endemic areas where electricity is not always available.

As a solution, we have focused on urinary tests to evaluate kala-azar activity. The appearance of proteinuria is occasionally reported by physicians in endemic areas, a phenomenon sometimes seen in tropical diseases, though it has not previously been evaluated as a possible indicator of disease activity. Recent research on urinary biomarkers has been concentrated in the field of acute kidney injury (AKI) [3], which is more broadly defined than acute renal failure [4]. The newly developed biomarkers can detect AKI much sooner than serum creatinine (SCr) or even blood urea nitrogen (BUN) testing. For instance, the increase of SCr is often detected 24–48 h after cardio-pulmonary bypass surgery, while the newly discovered urinary

biomarkers such as neutrophil gelatinase-associated lipocalin (NGAL), L-type fatty-acid-binding protein (L-FABP or FABP1), and kidney injury molecule-1 (KIM-1), can detect AKI as soon as 2 h after surgery [5,6].

Because the kidney is the second major organ involved in blood circulation and mirrors the body's condition in the urine, sensitive urine biomarkers for AKI are potential candidates for indicating the subtle changes in human health seen in tropical diseases. The urinary biomarker NGAL is partly derived from neutrophils and, therefore, NGAL levels in both blood and urine often increase in the presence of various infectious diseases [7,8]. Using NGAL as a biomarker in endemic areas merits serious consideration. On the other hand, growing evidence demonstrates the usefulness of fatty-acid-binding proteins (FABPs) as indicators of ischemic conditions in various organs. FABP1 is a potential predictive marker found in urine [9]. In addition, FABP1 can detect cisplatinum(CDDP)-induced nephrotoxicity [10] and contrast-medium-induced nephropathy [11] earlier than SCr or BUN. KIM-1 levels in urine are elevated in AKI somewhat later than NGAL and FABP1 but sooner than SCr. However, recent reports have made clear that KIM-1 is an efficient reporter molecule for indicating various drug-induced nephrotoxicities [12].

These highly sensitive urinary biomarkers may contribute not only to the monitoring of disease activity in tropical diseases including kala-azar, but also of drug-induced nephrotoxicities, a quite serious issue in kala-azar therapy. The therapy for eukaryotic microorganisms and parasites is difficult because the cellular structures of these organisms are more closely related to those of human beings than in the case of bacteria and viruses. FABP1 is one of the most-studied urinary biomarkers in AKI. Now we are introducing it as a tool for the control of kala-azar.

In this chapter, we review the basic attributes of FABP1 in the human kidney, report on our recent study of kala-azar patients using the newly-developed urine dipstick test based on immunochromatography and enzyme-linked immunosorbent assay (ELISA), discuss the specific applicability of FABP1 in the treatment of kala-azar, and suggest an additional AKI biomarker, interleukin(IL)-18, for future clinical studies related to the control of kala-azar.

8.2 Physiological and Pathophysiological Attributes of FABP1 in the Human Kidney

FABPs are intracellular lipid chaperones that transport fatty-acids to specific organelles. However, little is known about their exact biological functions and mechanisms of action [13]. FABPs are found in many different species including *Drosophila melanogaster*, *Caenorhabditis elegans*, mice, rats, and humans, and their tissue distribution of FABPs is rather ubiquitous [14]. Nine different types of FABP have been reported, including liver, intestinal, heart/muscle, adipocyte, epidermal, ileal, brain, myelin, and testis. In analyzing the human kidney, Veerkamp et al. found two types of FABPs in renal tubular cells: FABP1 or L-FABP, and heart/muscle type (FABP3 or heart-type FABP) [15]. They investigated the characteristics of renal FABP1 and FABP3 in detail. Renal FABP1 and FABP3 showed the same Kd values for liver and heart oleic acid. Compared with FABP1 expressed

in the liver, kidney FABP1 showed a more neutral isoelectric point (pI 5.8) and had two additional tryptophan residues. Therefore, renal FABP1 appears to be kidney-specific and could be a new subtype of FABP1.

The localizations of FABPs in the human kidney are predominantly expressed in the epithelial cells. FABP1 is specifically localized in the proximal tubular cells, and FABP3 in the distal tubular cells. Immunohistochemical analysis may show a weak positive staining in the parietal glomerular epithelial cells. FABP1 is a 14-kDa protein of beta sheet structure shaped like a clam shell (Fig. 8.1), and the promoter region of FABP1 contains binding sites for hepatocyte nuclear factor, hypoxia-inducible factor 1, and the peroxisome proliferator-activated receptor. Because of the anatomical structure of the kidney, the outer medullary region can easily be injured by hypoxia resulting from decreased peritubular capillary blood flow and subsequent oxidative stress in the renal ischemia/reperfusion model [16–18]. Proximal tubules are more susceptible to hypoxic stress than distal tubules. Under hypoxic conditions, proximal tubules tend to undergo necrosis, while distal tubules are likely to display apoptosis under the same level of hypoxic stress [19]. The FABP1 gene is responsive to hypoxic stress because its promoter region has a hypoxia response element.

In order for FABP1 to be excreted into the urine, a simultaneous decrease of peritubular capillary blood flow should be required. To prove this, we examined the peritubular capillary flow in a transplanted kidney and compared that with the level of urinary FABP1 concentration derived from the ureter of the transplanted kidney. Compared with the other urinary indicators measured, such as N-acetyl-β-D-glucosaminidase (NAG), alpha-1-microglobulin, beta-2-microglobulin, urinary FABP1 showed a significant correlation with the degree of peritubular capillary blood flow. When the ischemic time was defined from the harvest point from the donor to the time of the first urine-draining from the ureter of the transplanted kidney, the ischemic time correlated extremely well with urinary FABP1 (Fig. 8.2). One-hour protocol biopsy demonstrated that the origin of the urinary FABP1 was the proximal epithelial tubular cells shed to the tubular lumen.

Fig. 8.1 Crystal structure of human FABP1. FABP1 binds free fatty acids and their coenzyme A derivatives, bilirubin, and some other small molecules including HNE in cytoplasm, and is therefore considered to be involved in intracellular lipid and lipid peroxidation product transport. The image was obtained from Protein Data Base

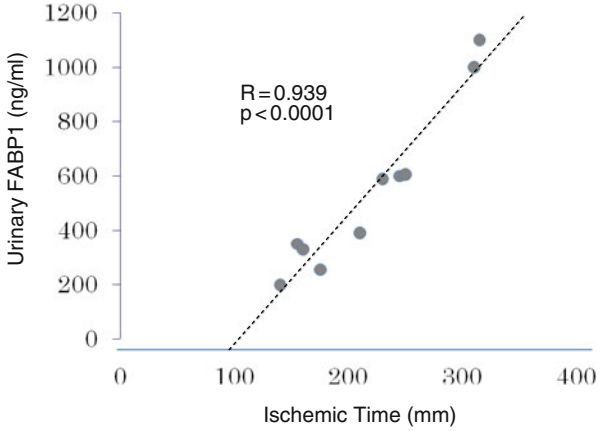

Fig. 8.2 Correlation between ischemic time and urinary L-FABP1. The ischemic time in living-related renal transplantation was defined as the period between the time point of clamping the donor's renal artery and the time point of appearance of virgin urine from the recipient's ureter (Reproduced with permission from the American Society for Nephrology.) [40]

The mechanism of FABP1 projection from the proximal cellular cytoplasms to the tubular lumen is not yet well defined. The physiological role of FABP1 is related to the metabolism of fatty acids that often bind to albumin. The fatty acid-bearing albumin is filtered through the glomerular basement membrane and then reabsorbed from the proximal tubular epithelial cells. FABP1 captures fatty acids and distributes them to cellular organelles such as mitochondria, lysosomes, peroxisomes, and so on for their energy metabolism (Fig. 8.3, right side). When proximal epithelial cells are subjected to injury – presumably as a result of decreased peritubular blood flow – intracellular oxidative stress will be increased by the breakdown of cytoplasmic membrane structures occurring largely as a product of lipid peroxidation. Because of the flexibility of the recognition site of FABP1, highly cytotoxic aldehydes (4-hydroxy-2,3-nonenal [HNE], 4-hydroxy-2-hexenal, etc.) can be bound to FABP1 and, presumably, excreted to the tubular lumen as a function of FABP1's role as chaperone for fatty acids and related products (Fig. 8.3, left side). The latter part of this explanation is plausible, but the details of the mechanism have not yet been clearly defined.

In human pathophysiological conditions, as we have previously reported, urinary FABP1 proved useful for the early detection of AKI after pediatric cardiopulmonary bypass surgery (CPB-AKI), where urinary FABP1 was examined at 4 and 12 h after the surgery [6]. Receiver-operating-characteristic (ROC) curve analysis of urinary FABP1 for CPB-AKI diagnosis was performed. The area under the ROC curve of urinary FABP1 at 4 h post-surgery was 0.810, which is an acceptable level for this single predictive biomarker. Univariate logistic regression analyses showed that both bypass time and urinary FABP1 were significant independent risk indicators for AKI.

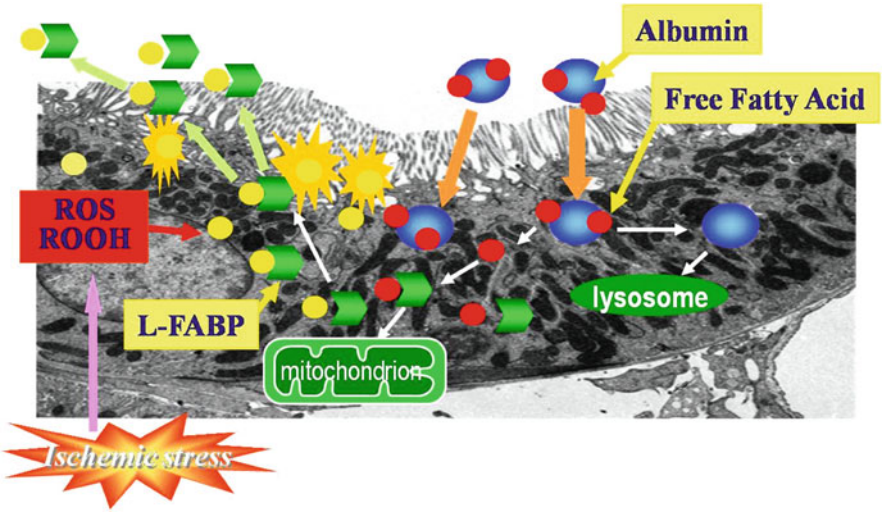

Fig. 8.3 Conceptual schema for the renal FABP1 mechanism. In the kidney, albumin filtered from glomeruli is reabsorbed predominantly in proximal tubules together with free fatty acids under physiologic conditions. After reabsorption, cytosolic albumin was transferred to lysosome, and fatty acid was released and received by FABP1 (L-FABP) during this process. Fatty acid-bound FABP1 will usually be relocated to cytosolic peroxisome for size reduction of fatty acids. Under ischemic conditions, lipid peroxidation products will accumulate in the proximal tubules and damage the proximal tubules (*left*). FABP1 is presumably capable of binding these noxious lipid peroxidative products and transferring them to urinary spaces. FABP1 is excreted from the proximal tubules into urine by binding cytotoxic lipids. ROOH denotes hydroperoxide radicals (Reproduced with permission from the American Society for Nephrology.) [40]

Pediatric cardiac surgery is recognized as an ideal clinical setting for the initial studies in biomarker development in terms of minimal comorbidity and known timing of renal injury [20]. Further studies are necessary for adult CPB-AKI.

Sepsis is the leading cause of death in critically ill patients, and the incidence of sepsis is increasing [21,22]. Sepsis causes AKI, and patients with both sepsis and AKI show an especially high mortality rate [23]. In one large multicenter study involving 30,000 critically ill patients, 50% of AKI cases were associated with septic shock [24]. Therefore, early prediction of sepsis-induced AKI will enable us to improve patient survival.

A recent study of septic shock patients showed urinary FABP1 levels in these patients to be significantly higher than in healthy subjects. In a cohort of 145 septic shock patients, a logistic regression analysis that included urinary FABP1, blood endotoxin level, c-reactive protein, peripheral white blood cell count, urinary NAG, and SCr revealed that only urinary FABP1 levels showed a significant association with patient survival rates. There was no correlation between SCr and urinary FABP1. Forty septic shock patients in another cohort were treated by polymyxin B-immobilized fiber (PMX-F) hemoperfusion. PMX-F treatment has been performed to treat severe sepsis in more than 30,000 patients in Japan since 1994, and a

meta-analysis demonstrated its efficacy in septic shock treatment [25]. Of 40 septic patients, 28 survived and 12 died. Among the survivors, urinary FABP1 levels were reduced by treatment. However, the non-survivors showed higher urinary FABP1 levels with a smaller decrease after the treatment compared with the survivors. These results suggest that urinary FABP1 levels might be able to reflect the severity of sepsis, and also to monitor the effectiveness of treatment [26].

Renal toxicity is a major concern in kala-azar therapy, both with traditional and newly developed drugs. The urinary indicator FABP1 has the potential to provide an early sign of the onset of nephrotoxicity, as was recently demonstrated in animal experiments. We have demonstrated that urinary FABP1 can detect interstitial changes after the administration of cyclooxygenase 2 (COX-2) inhibitors under a low-sodium diet [27]. Low sodium enhanced renin-angiotensin-aldosterone activity and reduced peritubular capillary blood flow. The additional administration of the COX-2 selective inhibitor meloxicam decreased peritubular capillary blood flow significantly. Mild interstitial fibrotic regions and partial cell infiltration were confirmed by histological examination. A more suggestive finding of this study was the relevance of the concepts responder and non-responder for the evaluation of drug toxicology. Celecoxib, another COX-2 inhibitor, induced low peritubular capillary blood flow in a certain fraction of animals (about 50%) that were categorized as responders. Animals of the responder group showed higher urinary FABP1 compared with the non-responders. When kidneys were harvested 4 weeks after celecoxib administration, kidneys derived from responders showed larger interstitial fibrotic areas compared with the non-responders (Fig. 8.4).

This sort of individual variation in phenotype is frequently encountered in human clinical studies. It is crucial to distinguish between drug responders and non-responders to improve treatment strategies and reduce adverse side effects of drug therapy. Urinary FABP1 may be able to flag responders at risk of drug renal toxicity. The carry-over of renal toxicity to chronic kidney diseases (CKDs) or that of AKI to CKDs has not been well-studied in the field of nephrology. However, urinary FABP1 will potentially be able to serve as an early warning sign for such carry-overs, given its proven usefulness as an indicator of various CKDs [28].

Focal glomerulosclerosis (FGS) is a significant cause of primary glomerulonephritis. Its incidence appears to be increasing especially among the African-American population. Drug treatment, including glucocorticoids and calcineurin inhibitors, sometimes fail to produce the desired therapeutic response leading to remission. When urinary FABP1 was measured in FGS, the level was significantly higher than minimal change (10.2 ± 8.4 μg/g Cr; $n = 24$) or healthy control (7.4 ± 4.2; $n = 20$). The drug-resistant FGS group showed significantly higher urinary FABP1 levels (122 ± 78.4; $n = 8$) compared with the sensitive group (45.9 ± 32.0; $n = 9$) [29].

These observations in CKD suggest that the target population suited for intensive treatment should exhibit the features of lower peritubular capillary blood flow, subsequent hypoxic conditions, and the presence of interstitial fibrosis, all of which can be monitored by urinary FABP1. Recently, Nakamura et al. reported a new L-type calcium channel blocker that decreased both urinary protein and FABP1 in mild

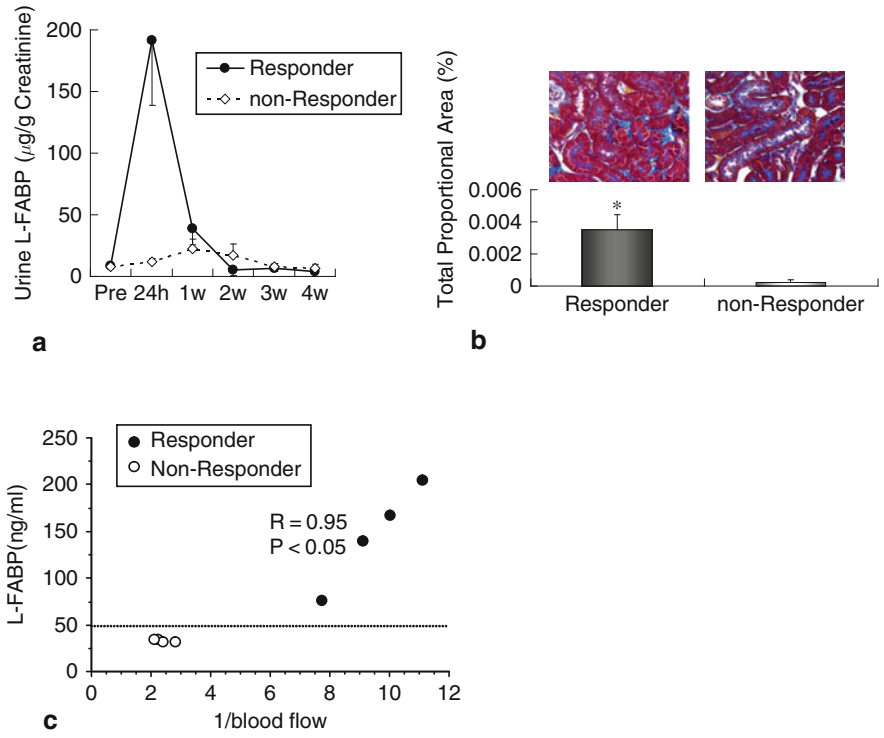

Fig. 8.4 Analyses of urinary FABP1 (L-FABP) responders and non-responders after starting celecoxib. **a** Time course of urinary FABP1 levels. Animals excreting urinary FABP1 > 50 μg/g creatinine during the follow-up period were defined as responders ($n = 4$). Those excreting lower levels were defined as non-responders or low responders ($n = 4$). **b** Representative images obtained from responder and non-responder kidneys. Quantitative analyses were performed for interstitial fibrosis. Asterisk depicts $p < 0.05$. **c** Correlations between urinary FABP1 and peritubular capillary blood flow 2 days after starting celecoxib (Reproduced with permission from S. Karger AG.) [27]

CKD patients complicated with hypertension [30]. They also reported that urinary FABP1 could detect histological improvement by angiotensin-converting enzyme inhibitor and angiotensin II receptor blocker combination treatment in normotensive IgA nephropathy [31].

8.3 Potential of FABP1 as a Urinary Diagnostic Tool in Kala-Azar

In our cross-sectional study, urinary FABP1 was examined in both 162 kala-azar patients (median age, 18.0 years [range, 5–60]; male:female = 89:73) and 46 healthy control individuals (median age, 24.5 years [range, 7–50]; male:female = 30:16). Kala-azar was diagnosed by rK39 and spleen biopsy exam, and only cases with a clear diagnosis of kala-azar were included. The patients' urine was obtained at the initial clinical visitation and measured for urinary creatinine, protein, NAG,

Table 8.1 Urinary L-FABP (uL-FABP) and protein (uProtein) levels in both kala-azar and EC cohorts in Mymensingh, Bangladesh

		N	Mean	SD	SE	<95% CI	>95% CI	Prob>|t|
uL-FABP	Kala-azar	162	113.41	223.30	17.54	78.77	148.06	
(ng/mL)	EC	46	0.94	1.95	0.29	0.36	1.52	< 0.0001
uProtein	Kala-azar	162	35.83	46.01	3.62	28.69	42.97	
(mg/dL)	EC	46	3.41	2.22	0.33	2.76	4.07	< 0.0001
uL-FABP	Kala-azar	162	153.20	326.59	25.66	102.53	203.87	
(μg/g Cre)	EC	46	1.93	3.85	0.57	0.78	3.07	< 0.0001
uProtein	Kala-azar	162	486.56	510.66	40.12	407.33	565.79	
(mg/g Cre)	EC	46	125.48	164.69	24.28	76.57	174.38	< 0.0001

and FABP1. Because these indicators do not directly explain the etiology of kala-azar, they may reflect the severity of the infection and serve as indicators of kidney stress.

The summary data in Table 8.1 demonstrate that both urinary FABP1 and protein levels were significantly increased in the kala-azar cohort, compared with the control group. When these urinary concentrations were corrected by urinary creatinine, the values for the kala-azar group were increased from that of the control. Presumably, the reason for this is that SCr levels in residents of endemic areas are lower than those of individuals in the West and in Japan because of the widespread malnutrition in the endemic areas [32]. The values of urinary NAG in both the kala-azar and control cohorts often lay below the detectable limit. Although samples were stored below −20°C, high temperatures in the endemic areas and transfer time between sites may have affected the urinary indicators. NGAL data can be expected to be similarly affected, as NGAL requires even more careful handling of samples [33]. FABP1, on the other hand, is a stable protein in the urine, and Fig. 8.5 demonstrates this stability by showing a comparison of values measured immediately after sampling with those of samples stored at −20°C, 4°C, and room temperature (RT) for periods of 6, 24, and 48 h. Urine stored at RT for 48 h showed concentrations within 20% of the initial measurements. In addition, FABP1 levels did not change according to gender or daily fluctuations. Based on this information, the cut-off value of FABP1 for immuno-chromatography was set at 100 ng/mL. Proteinuria was measured by the Bradford method (Bio-Rad Laboratories; Hercules, California, USA) with the standardization of bovine serum albumin, but the potential cut-off value was too low to be distinguishable by protein dipstick tests.

Among a cohort of 162 kala-azar patients, we were able to monitor the urine and blood serum of 50 patients (median age, 17.5 years [range, 5–45], male:female = 29:21) over 3 months, including 4 weeks of in-hospital treatment with sodium stibogluconate (SSG). This was a cooperative operation with Dr. Kazi M. Jamil and his colleagues at the International Centre for Diarrhoeal Disease Research, Bangladesh, where the ethical review committee approved this study. The duration of fever was 85.8 ± 32.7 days. Fifty-eight percent of patients showed abdominal swelling, especially on the left side, and 94% had a history of weight loss. A family history

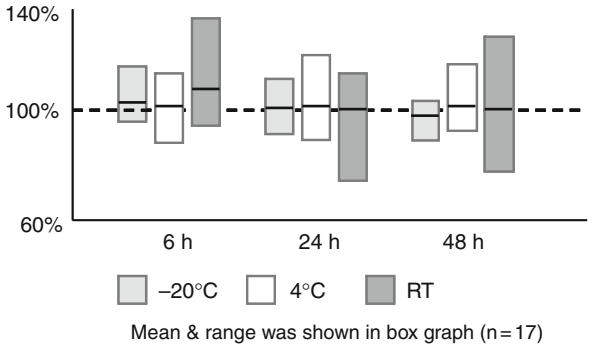

Fig. 8.5 Urine storage condition and urinary FABP1. Box graphs demonstrate the range of FABP1 (maximum and minimum values) and the thick bar depicts mean value of each group. The reproducibility of urinary FABP1 was the best when stored at −20°C. When urines were stored in RT, that range of 6-h samples was comparable to that of 48-h samples. Mean FABP1 levels were virtually the same in all conditions

of kala-azar was found in 82% of patients. Because of the risk associated with spleen biopsy, inclusion criteria were as follows: (a) hemoglobin above 6.0 g/dL; (b) platelet count above 5×10^4 cells/μL; (c) prothrombin time less than 4 s. The diagnosis of kala-azar was confirmed in all patients via both spleen biopsy and rK39 testing. All patients in the study clinically displayed complete remission 4 weeks after starting therapy. SCr and BUN remained at almost the same levels during the follow-up period (Fig. 8.6a). In contrast, the elevated urinary FABP1 levels seen during the initial clinical visitation decreased within 1 week after starting therapy, which presumably reflects the efficacy of the prescription (Fig. 8.6b). A similar trend was seen in proteinuria levels. When the results in the 50 cases in the follow-up study were stratified by urinary FABP1 levels, the cut-off value of 10 ng/mL might provide a more sensitive indicator of disease activity and the efficacy of the prescription (Fig. 8.6c).

8.4 Efficacy of Detecting Renal Toxicity by Urinary FABP1

The potential use of urinary FABP1 for detecting renal toxicity was already reported in studies of CDDP-AKI in animals [10] and in human contrast-medium-induced AKI [11]. We decided to study the use of FABP1 to monitor amphotericin-B-induced kidney injury through lowered nephron levels in patients treated with amphotericin B, which can cause injury when concentrated in the urine. Amphotericin B binds to the sterol in the membranes of renal tubular epithelial cells and endothelial cells, and modifies membrane permeability because amphotericin B, acting as a pseudo-phospholipid, interacts with sterol molecules to cause the formation of aqueous pores consisting of an annulus of polyene and sterol, with the hydrophilic region of the drug molecule facing the interior of the pore. This mechanism is more or less inevitable when starting treatment with amphotericin B

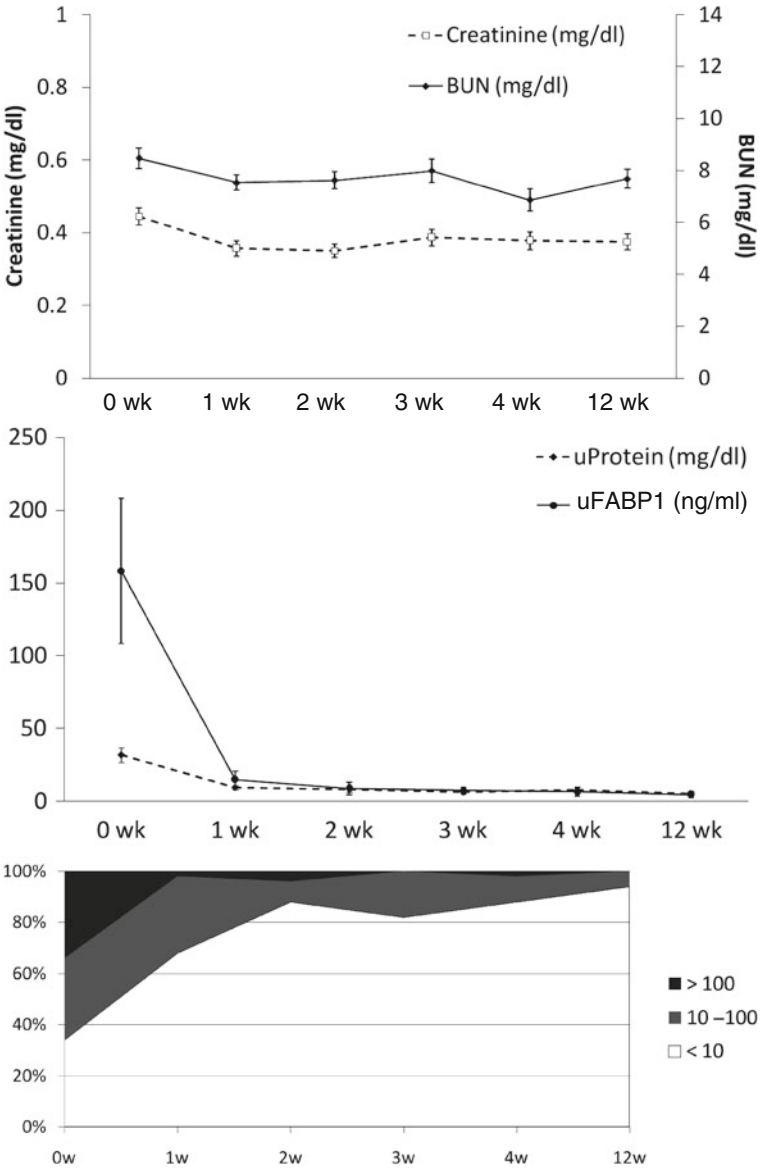

Fig. 8.6 Follow-up data on 50 kala-azar individuals. The diagnosis of kala-azar in all 50 patients was confirmed by spleen biopsy and rK39. Sample correction was performed immediately before treatment and at 1, 2, 3, 4, and 12 weeks after treatment with SSG. **a** SCr and BUN (serum). **b** Urinary FABP1 and protein. **c** Follow-up data was stratified by urinary FABP1 level in each sampling point

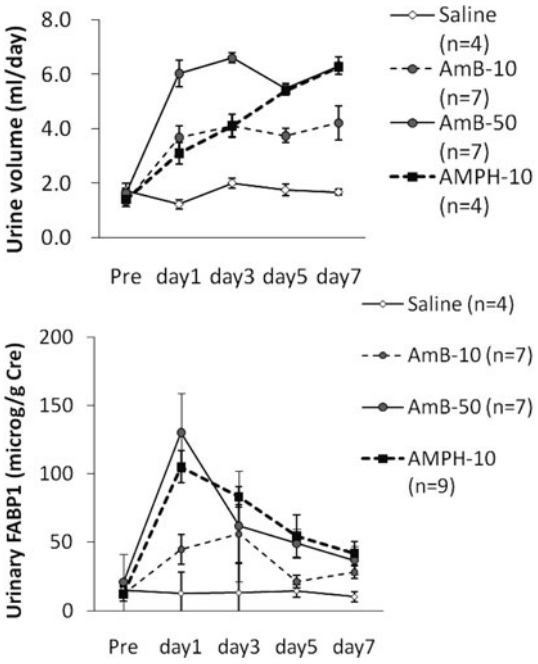

Fig. 8.7 Amphotericin B and Ambisome toxicity were examined using humanized FABP1 transgenic mice. Both drugs were injected in each animal every day of the indicated dose (mg/kg). Urine samples were serially collected in glass-made metabolic cages. Mean and SE values are demonstrated in the graph. The dose in this experiment is higher than that used in previous studies. When animals were subjected to amphotericin B 50 mg/kg, all were dead within 24 h. **a** Urinary volume. **b** Urinary FABP1 level

and related drugs, but toxic doses induce acute spasms of the renal vasculature and can cause severe renal injury [34]. Therefore, we expected that urine FABP1 could be used to identify susceptible individuals among the patients being treated with this drug.

To examine the toxicity of amphotericin B and liposome-conjugated amphotericin B (Ambisome), different dosages, as shown in Fig. 8.7, were injected once every 24 h to humanized FABP1 mice [35]. When the animals were subjected to amphotericin B at a dose of 50 mg/kg, all were dead within 24 h. Ambisome 50 mg/kg was tolerated by the animals, but urine volume increased three-fold within 24 h. When the animals were subjected to amphotericin B at a dose of 10 mg/kg, urine volume increased daily and reached three times the original volume within a week. However, Ambisome at 10 mg/kg increased urine volume more moderately compared with amphotericin B at 10 mg/kg. Within 24 h after starting these treatments, urinary FABP1 clearly reflected the level of injury demonstrated by urine volume as a clinical symptom. However, urinary FABP1 levels started to decrease after 24 h even when the drug injections continued.

The reason for this partial increase of FABP1 is not yet clear. Because the renal localization of FABP1 is limited to the proximal tubular cells [35], the effect of amphotericin B on proximal tubules is presumably limited at higher dosages when the peritubular blood flow is affected by the initiation of therapy. Nonetheless, these increased dosages have gradually been adopted. Recently, higher doses of Ambisome and other medications have been considered to increase patient compliance. In such cases, urinary FABP1, particularly the level 24 h after initiation of

therapy, can serve as a triage biomarker to monitor the safety of the treatment for individual patients.

8.5 Development of the Immunochromatography (Dipstick) Method for Measuring FABP1

More easily applicable testing methods are needed for use particularly in outlying regions in the endemic areas. While the ELISA method has its advantages, our new convenient dipstick test for urine is more suitable, even for use in qualitative studies. We developed the dipstick test using the immunochromatography method; the mechanism is explained in Fig. 8.8. This method facilitates the original sandwich ELISA method of FABP1 in an immuno-dipstick test. Approximately 90 μL of urine is applied to the dipstick using a small eyedropper and allowed to sit for 10 min. The cut-off value for the most recent FABP1 dipstick is currently 100 ng/mL. A run of the urine sample is considered successful when the test band is approximately as dark as the control band. We tested the dipstick at the Kala-Azar

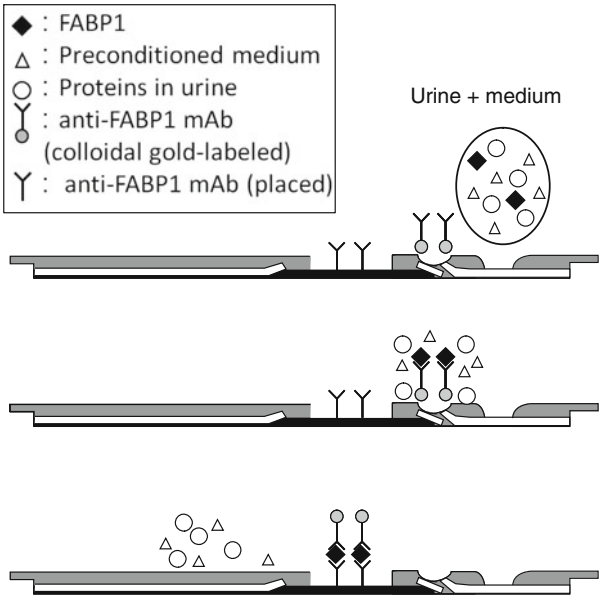

Fig. 8.8 The principle of immunochromatography test for urinary FABP1. For the purpose of kala-azar screening, the cut-off value of 100 ng/mL was tentatively assigned, and the siliconized gold-colloid filter (*gray circle* with antibody mark) was optimized for endemic urine. From top to bottom schema, applied urine will run to left side and colorimetric reaction will appear as the single band. *Black rectangle, white triangle*, and *white circle* are FABP1, pretreatment agent, and contaminant proteins, respectively. FABP1 is captured by fixed anti-FABP1 antibody and color will appear by the gold-colloid effect

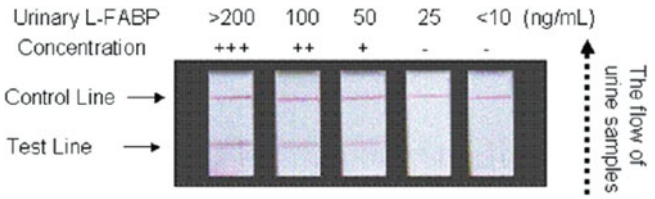

Fig. 8.9 Tone level list of urinary FABP1 immunochromatography test (FABP1 dipstick). Dip-score was determined by tone level list

Research Center in Bihar, India, with Dr T. K. Jha and his team in 2008. We had a chance to examine 25 patients. As shown in Fig. 8.9, the scale of color tones for the result band reflects a range of sample concentration from (−) to (3+), and the scale of tones has been assigned the digits 0–4. The actual distribution of urine samples is plotted in Fig. 8.10a, showing the comparison between ELISA and dipstick data (the dip score), and further summarized in terms of dip score average in Fig. 8.10b. Both exhibit extremely good correlation as expected.

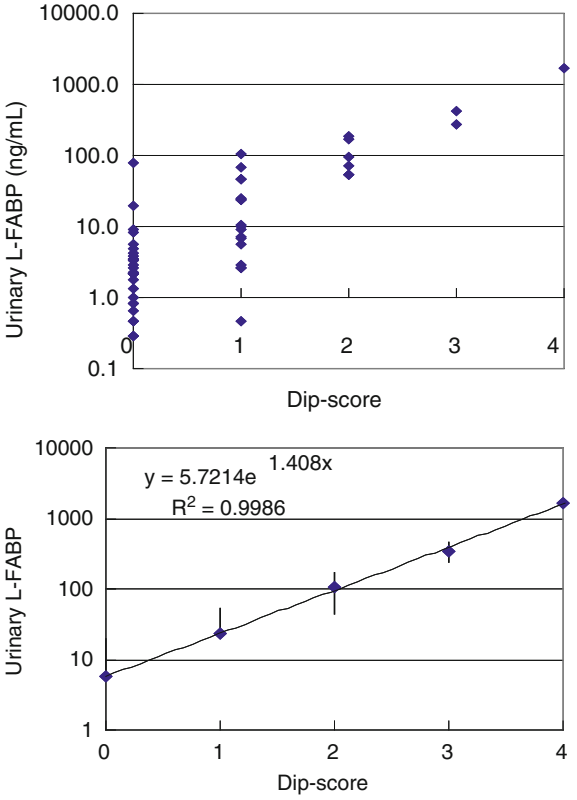

Fig. 8.10 Comparison of urinary FABP1 dip-score versus ELISA. Note: The *y-axis* of ELISA uses logarithmic scale. **a** The distribution of each sample data. **b** The average of each dip-score was summarized in line graph

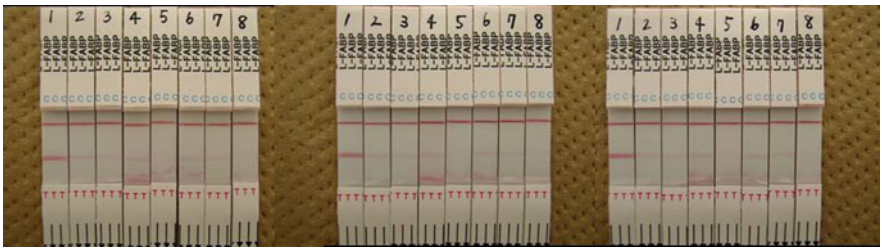

Fig. 8.11 Within-run reproducibility test. Tests were examined three times on different date using pre-measured samples and standard FABP1-dependent positive and negative controls

The within-run reproducibility was examined three times on different dates using pre-measured samples and standard FABP1-dependent positive and negative controls. These reproducibility tests gave quite excellent results (Fig. 8.11). The gold-colloid filter in the dipstick cassette had to be adjusted to achieve optimum urine test results in the endemic area, and different types of siliconized filters were tested to reduce oozing and increase contrast. The quantification of the dipstick test was examined using a commercially available compact battery-powered immuno-reader (Fig. 8.12). ROC analysis for detection was examined by dip-score and immuno-reader, and these results were compared with the corresponding ELISA results. Independent samples collected in the endemic area were as follows: 25, 20, and 17 individuals in the control cohort; 74, 79, and 82 individuals in the positive cohort. The area under the ROC curve of the dip-score showed 0.78, 0.87, and 0.89, and that of the immuno-reader showed 0.81, 0.88, and 0.90 (Fig. 8.13).

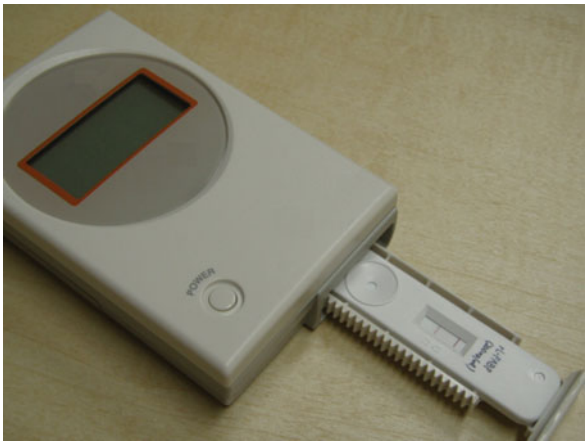

Fig. 8.12 Commercially available compact immuno-reader

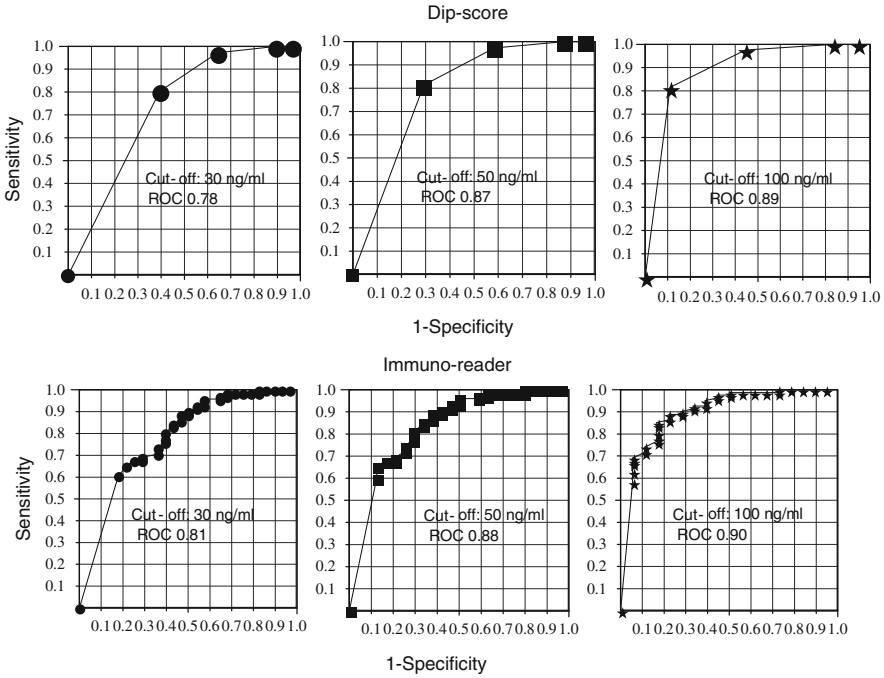

Fig. 8.13 ROC analyses determined by FABP1 ELISA were compared to dip-score or immuno-reader results. From the *left panel*, the cut-off values assigned were 30 ng/mL (positive individuals, 25; negative individuals, 74), 50 (positive, 20; negative, 79), and 100 ng/mL (positive, 17; negative 82)

8.6 IL-18 as Another Potential Urinary Biomarker in Kala-Azar

IL-18 is produced by a variety of cells such as macrophages, dendritic cells, and Kupffer cells. IL-18 that induces production of interferon-gamma (IFN-γ) is associated mainly with the induction of T helper cell type 1 (Th1) response, though IL-18 can modulate T helper cell type 2 (Th2) responses and the production of IL-4, IL-13, and IL-10 under limited conditions.

Human susceptibility to kala-azar infection involves the induction of both Th1 and Th2 responses, but the ability to control infection is associated with a down-regulation in IL-10 production and an upregulation of IFN-γ and IL-12 production. IL-12 is a potent inducer of IFN-γ production when used as single stimulus [36]. It is assumed in human infection with kala-azar that the balance between Th1 and Th2 responses is important in controlling resistance and/or susceptibility, but the detailed mechanism in kala-azar has not yet been elucidated. IL-18 is the enhancer of IL-12 induced IFN-γ production. It is generally accepted that IFN-γ is needed for the control of and protection from *Leishmania* infections.

In kala-azar, development of the disease and control of the infection depend on the effectiveness of IFN-γ-induced innate and adaptive cellular immune responses,

which affect intracellular killing by activated macrophages. Therefore, the increase of both upstream and downstream cytokines involved in the release of IFN-γ was observed in kala-azar patients' plasma before starting treatment [37]. These increases were reduced after starting treatment for kala-azar. Because of the shortage of parameters to evaluate appropriate disease control strategies for kala-azar, as mentioned in our earlier discussion, these findings provide important information. On the other hand, it is extremely difficult to draw blood and perform sophisticated laboratory examination on the ground in endemic areas. Obtaining information on the cytokines upstream or downstream of IFN-γ in the form of urine samples would make this information more feasible for use for disease control purposes.

Urinary IL-18 detection is successful in human AKI [38] and, therefore, urinary IL-18 may detect systemic upregulation of IL-18 in kala-azar. Because the disease activity of kala-azar is mainly derived from macrophages with Leishman-Donovan bodies, and macrophages are the source of IL-18, the follow-up of IL-18 should be parallel to the disease activity of kala-azar and useful for monitoring the efficacy of kala-azar therapy.

It is necessary to evaluate urinary IL-18 to determine its potential for distinguishing the kala-azar cohort from the endemic control (EC) group. Urinary IL-18 of 135 kala-azar cases (diagnosis made by spleen biopsy and rK39) was compared with that of an EC cohort of 42 individuals. Figure 8.14 demonstrates the significant increase of urinary IL-18 in the kala-azar cohort immediately before starting therapy. Follow-up testing of IL-18 was performed in 23 of the 50 kala-azar patients whose cases were followed for FABP1 levels for 12 weeks, as discussed in Section 12.3 above. Because urine samples displaying higher FABP1 levels required repeated measurements at higher dilutions, fewer residual samples were available for IL-18 testing, which explains why the sample number used for IL-18 measurement is lower than that for FABP1. As discussed above, SCr and BUN levels remained virtually constant over the 12-week follow-up period, and did not correlate with either disease activity or therapeutic efficacy (Fig. 8.6a). In Fig. 8.14, urinary IL-18 in kala-azar

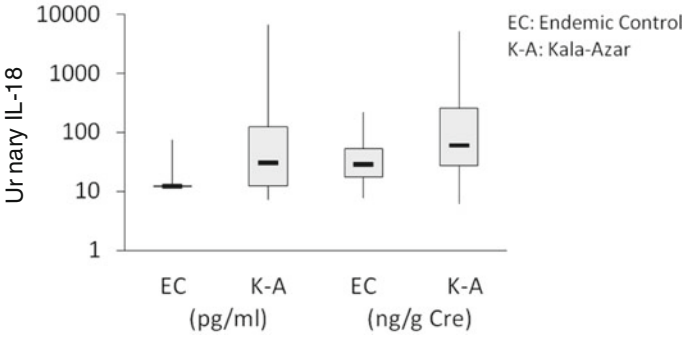

Fig. 8.14 Comparison of urinary IL-18 levels between EC patients ($n = 42$) and kala-azar patients (K-A; $n = 135$)

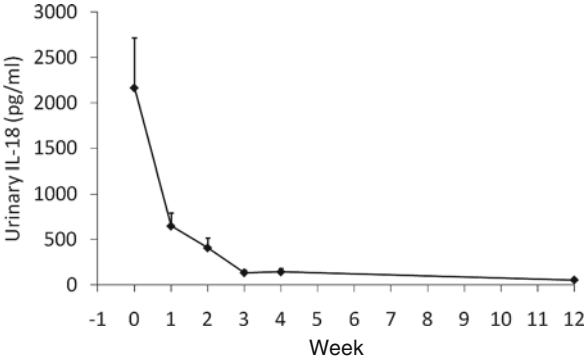

Fig. 8.15 Follow-up data on urinary IL-18 in kala-azar patients ($n = 23$). IL-18 levels are described as mean ± SE

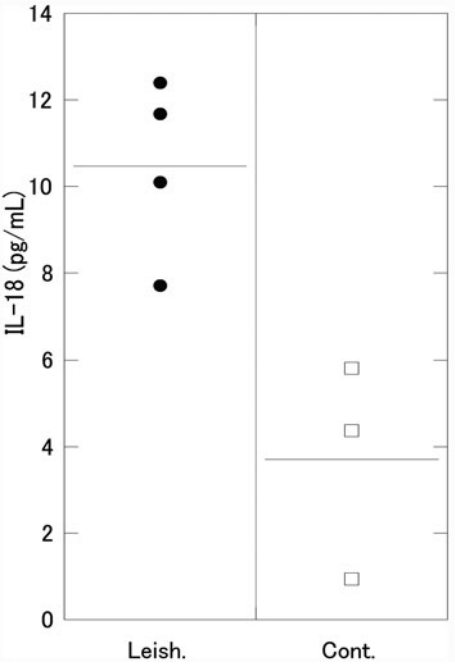

Fig. 8.16 Efficacy of urinary IL-18 for the detection of disease activity in mice kala-azar model. Urinary IL-18 of mice kala-azar (Leish) and controls were demonstrated ($n = 4$ in each group). Mice kala-azar was established with *L. donovani*, which demonstrated positive rK39 in serum and PCR in buffy coat

patients showed a wide variety of values before treatment based on the severity of individual cases, which can vary a great deal, as can been seen from the standard deviation in the duration of fever. All 23 of the patients studied received standard treatment with SSG. Figure 8.15 plots urinary IL-18 levels against time. The initial rise of urinary IL-18 decreased after patients started SSG treatment. This treatment was continued for 4 weeks, during which time the level of urinary IL-18 normalized. Signs of relapse were not clinically apparent until 12 weeks, at which time IL-18 levels were virtually constant or even normalized.

The efficacy of urinary IL-18 for the detection of disease activity was evaluated in mice infected with visceral leishmaniasis for 54 weeks (mice kala-azar model). The *Leishmania donovani* infection was confirmed using polymerase chain reaction (PCR) for buffy coat of white blood cells and rK39 ELISA for serum. Figure 8.16 demonstrates the significant increase of urinary IL-18 in infected mice ($n = 4$) compared with the urine of control mice ($n = 4$). This observation demonstrates that urinary IL-18 can serve as an indicator of disease activity in leishmaniasis, similarly as in kala-azar.

In summary, the significantly increased urinary IL-18 levels found in the kala-azar cohort before the start of treatment decreased after treatment had been initiated. IL-18 is one of the hallmarks of macrophage-induced cytokine storm as seen in kala-azar. Urinary IL-18 levels before and after kala-azar treatment can provide an excellent indicator of disease activity. The further development of the currently standard ELISA system in the form of immunochromatography makes testing of this sort much more feasible throughout endemic areas.

8.7 Future Prospects of Urinary Biomarker Use in Kala-Azar

Although we were able to conduct follow-up examinations of 50 kala-azar patients for 3 months, and will continue follow-up examinations until the end of the 12th month, almost all the patients responded well to SSG within 2 weeks after starting treatment. Urinary biomarkers are expected to reflect disease activity, but further studies are needed to provide data for intractable cases of kala-azar. Initial disease activity tends to resolve immediately with the appropriate treatment, but occasionally it is sustained and displays clinical symptoms. Sporadic disease activity of this sort can be monitored using urinary biomarkers, as demonstrated in Fig. 8.17 based on the study discussed above. The occurrence of drug-resistant

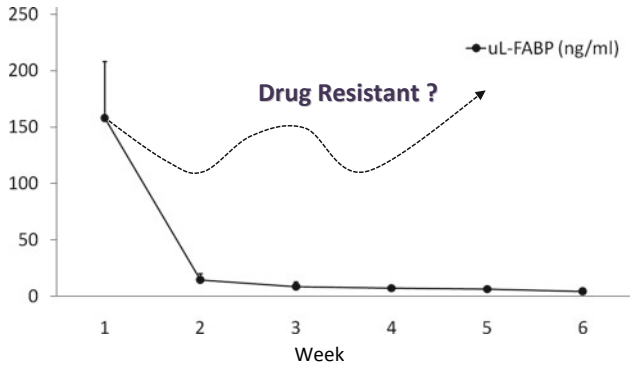

Fig. 8.17 A pattern showing urinary FABP1 efficacy in kala-azar. Urinary FABP1 may contribute to follow-up resistive cases in endemic areas

kala-azar has recently been recognized and is often reported in India [39]. Early detection of resistance will allow the treatment to be modified accordingly, which will benefit patients. To help achieve this, we are currently offering FABP1 tests in endemic areas in India, Nepal, and Bangladesh, where resistant cases are rapidly increasing. The initial urinary FABP1 measurement in these areas showed that a cut-off value for the positive dipstick evaluation validated by ELISA was 62.9 ng/mL. The area under the curve of ROC was 0.80 (as found Dr. S. Sundar and colleagues; unpublished data). A future study is necessary to clarify the efficacy of dipstick and ELISA as the diagnostic tests determining clinical action ability.

Acknowledgment This study was supported in part by the Special Coordination Funds for Promoting Science and Technologies (Grant no. 1200015), Ministry of Education, Culture, Sports, Science and Technology, Japan.

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Chapter 9
Mass-Survey Using Urine and Confirmation by LAMP for Control of Visceral Leishmaniasis

Makoto Itoh and Hidekazu Takagi

Abstract Finding asymptomatic carriers of *Leishmania donovani* is an important strategy for the control of visceral leishmaniasis (VL) or kala-azar. A strategy comprising two diagnostic methods is proposed. Mass screening with urine samples – which can be collected easily by non-skilled people with good compliance of residents – for examination of anti-*L. donovani* antibodies using enzyme-linked immunosorbent assay (ELISA) is the first step. The ELISA positives are those who had been or still are infected by the parasite. To confirm a present infection, the antibody positives are followed by loop-mediated isothermal amplification (LAMP), which detects the parasite DNA in blood samples, the collection of which is much safer than that of spleen or bone marrow aspiration used for conventional parasitological confirmation. The LAMP specificity and sensitivity are equivalent to that of nested polymerase chain reaction. Moreover, useful results are obtained within 1 h of isothermal incubation. The combined use of these diagnostic tools will contribute to the control of VL.

Keywords Visceral leishmaniasis · Urine ELISA · LAMP

Abbreviations

ELISA	Enzyme-linked immunosorbent assay
LAMP	Loop-mediated isothermal amplification
PCR	Polymerase chain reaction
VL	Visceral leishmaniasis

9.1 Introduction

Visceral leishmaniasis (VL) is a vector-borne fatal systemic infection caused by various species of the *Leishmania* intracellular protozoan. It is considered a highly neglected disease [1] that typically strikes the poorest people in rural areas: those

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_9,

who are least able to afford treatment. More than 60% of the world's VL cases are reported from Bangladesh, India, and Nepal. An estimated 150 million people are at risk and 40,000 new cases of VL are reported annually [2]. The disease is usually fatal if left untreated. The number of VL cases has been underestimated because only passive surveillance systems are used currently [3]. The lack of a simple but reliable diagnostic method might constrain the employment of an active surveillance system. Aldehyde tests have been used widely because of their simplicity, but they have low specificity to VL. Subsequently, enzyme-linked immunosorbent assay (ELISA) and direct agglutination test, which detect specific antibodies to *Leishmania* antigens in serum samples, were used. However, the need for expertise limits their use. Recently, a dipstick test kit was developed that can detect antibodies in serum samples to a recombinant antigen, rK39, part of *Leishmania chagasi* kinesin-related protein. It is now widely used because of its simple method and high sensitivity [4, 5]. An ELISA method that detects antibody in urine but not serum samples, using acetone-treated *L. donovani* promastigote antigen or a recombinant antigen rKRP42, reportedly has high sensitivity and specificity [6, 7]. The use of urine has been considered valuable for the diagnosis of VL because of patient compliance, ease of performance, and safe sample collection. These immunodiagnoses have high sensitivities but are unable to distinguish past from current infections.

Another type of kit, KAtex, detects the parasite-derived antigen in urine samples with a latex agglutination test [8]. A positive result indicates a current infection. A DNA detection method using polymerase chain reaction (PCR) was also developed to diagnose the current infection of VL by detecting parasite-derived DNA [9]. Collection of blood samples for PCR is much safer than spleen or bone marrow aspiration. Recently, loop-mediated isothermal amplification (LAMP), a method that amplifies DNA rapidly under isothermal conditions, was developed and showed high sensitivity [10, 11]. Initially, LAMP was applied to detect DNA from protozoans such as *Trypanosoma* species and *Plasmodium falciparum* [12–14]. More recently, a LAMP assay was also developed for the diagnosis of VL [15].

Using those newly developed tools, a strategy for the survey of VL is proposed (1) to detect sources of VL transmission; (2) to find new endemic foci of VL; and (3) to monitor VL cases. The strategy comprises two steps: mass survey with an antibody detection tool using urine samples as a first step, and confirmation of the active infection among the antibody positives using LAMP as the second step.

9.2 Mass Survey of VL Using Urine

The merit of using urine instead of serum as samples for diagnosis is its ease of collection, which will facilitate compliance of people in field activities. Untrained people can collect many samples in a short time, even at schools for example. It might be easier to gain the cooperation of asymptomatic subjects for urine samples than it would be for blood samples. To take advantage of this fact, several immunodiagnostic methods using urine have been established for other diseases such as filariasis [16] and schistosomiasis [17, 18]. Kohanteb et al[19] reported the

Table 9.1 Sensitivity and specificity of rKRP42 urine ELISA for the diagnosis of visceral leishmaniasis

Sample	VL	EHC	NHC	M	TB	CL	OD
Positives/negatives	108/115	0/59	0/80	0/58	0/13	0/23	1/7
Positive rate (%)	(94%)	(0%)	(0%)	(0%)	(0%)	(0%)	(14.3%)

VL, visceral leishmaniasis; EHC, healthy controls from endemic areas; NHC, healthy controls from non-endemic areas; M, malaria; TB, tuberculosis; CL, cutaneous leishmaniasis; OD, other diseases (two amebic liver abscess cases, two aplastic anemia cases, aplastic anemia with nephritic syndrome case [showed positive result], an aortic stenosis case, and a viral fever case).

detection of soluble antigen and antibody in the urine of VL patients using double-countercurrent immunoelectrophoresis; de Colmenares et al[20] detected antigenic compounds in urine using Western blotting.

We developed ELISA systems for diagnosis of VL by detecting antibodies to *L. donovani* antigen in urine samples. The acetone-treated *L. donovani* antigen was used for the ELISA with a high sensitivity and specificity of 93.5 and 89.3%, respectively [6]. We subsequently developed an ELISA based on the recombinant protein rKRP42, which is part of an L. donovani kinesin-related protein and a homolog of rK39 [21]. When rKRP42 and rK39 were compared, the amino acid sequence of rKRP42 showed 89.3% identity and 98.7% homology with rK39; it has 39 more amino acids than rK39 has. The rKRP42 was validated with ELISA using urine samples [7]. The urine-based ELISA with rKRP42 showed 94% sensitivity and 99.6% specificity (Table 9.1). The sensitivity was similar, but the specificity was higher than that of the ELISA using the acetone-treated *L. donovani* antigen. A comparison of the rKRP42 urine ELISA with the commercially available urinary antigen detection kit (KAtex) using 108 VL samples showed much higher sensitivity in the ELISA (94.0%) than with the KAtex (55.6%). Therefore, the ELISA with rKRP42 antigen is a useful tool for mass surveys to find the most subjects with parasitic infection. Development of point-of-care test kits for use with urine is in progress to make the survey more convenient.

9.3 Confirmation of VL Using LAMP

For confirmation of VL, detection of an amastigote form of *L. donovani* in spleen or bone marrow aspirates has been used. This method, however, is invasive, laborious, and skill-intensive. Moreover, it is limited to well-equipped hospitals. Diagnostic methods based on PCR have become more popular for diagnosis of leishmaniasis [9, 22]. Peripheral blood, which showed high sensitivity, became popular for use as samples for PCR to avoid invasive procedures [9, 22, 23]. Nevertheless, PCR requires a well-established laboratory and equipment such as a thermal cycler and a system to analyze amplicons. Recently, LAMP was developed as a novel method to amplify DNA with rapidity and high specificity under isothermal conditions [11, 24]. The method consists simply of incubating a mixture of sample DNA and other

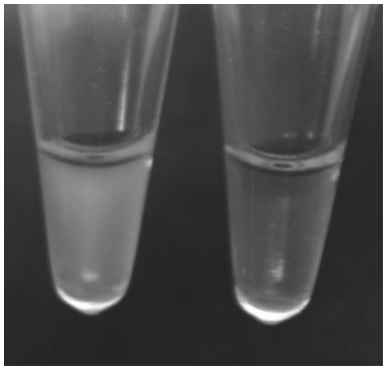

Fig. 9.1 Turbidity of reaction fluid produced by LAMP: *Left*, with target gene; *Right*, without target gene

reagents for 1 h at 60–65°C using simple equipment such as a heat block or a water bath. Moreover, as the LAMP reaction progresses, the reaction by-product (pyrophosphate ion) binds to magnesium ions and forms a white precipitate of magnesium pyrophosphate, which can be inspected visually (Fig. 9.1).

We developed the LAMP to detect *L. donovani* kinetoplast minicircle DNA [9]. The mixture of the primer set, each deoxynucleoside triphosphate, *Bst* DNA polymerase, and sample DNA was incubated at 65°C for 50 min using a heat block. The LAMP detected 1 fg of *L. donovani* DNA, thereby displaying ten times greater sensitivity than a conventional PCR performed according to an earlier report [25], and showing equivalent sensitivity to that of nested PCR performed according to a report [26] describing the use of extracted *L. donovani* promastigote DNA (Fig. 9.2). Specificity of LAMP was also confirmed: DNA samples from *L. infantum*, *L. major*, *L. mexicana*, *L. tropica*, *L. braziliensis*, and *Plasmodium falciparum* were LAMP negative. The DNA samples extracted from

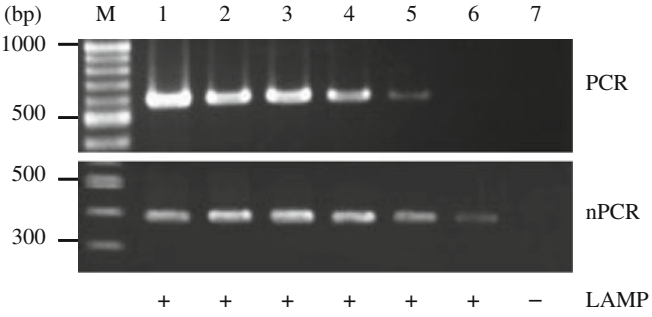

Fig. 9.2 Sensitivity of PCR, LAMP and nested PCR for detection of *Leishmania donovani* DNA. Agarose gel electrophoresis profiles of PCR (*upper*) and nested PCR (nPCR, *lower*) with the results of LAMP (indicated as positive "+" or negative "–"): lane M, 100-base pair ladder; lanes 1, 2, 3, 4, 5, and 6, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg of DNA, respectively; lane 7, negative control

venous blood samples (1 ml) from VL patients confirmed by spleen biopsy were examined using LAMP. Of them, 87% (52 of 60 samples) were judged as positive with LAMP, which was the same sensitivity as that of the nested PCR. Real-time monitoring of the amplified DNA using LAMP is possible by measuring the fluorescence of SYBR Green or the turbidity of magnesium pyrophosphate [12–14]. The serially diluted *Leishmania* DNA was subjected to the real-time LAMP using SYBR Green. The increase in the fluorescence of SYBR Green bound to the amplified double-strand DNA was measured using LineGene, a real-time monitoring fluorescence quantitative detection system, at every 1 min; the detection was linear over the range examined (Fig. 9.3). Although some special apparatus, such as a fluorescence detection system or turbidimeter, is required, real-time LAMP enables quantification of the target DNA in samples.

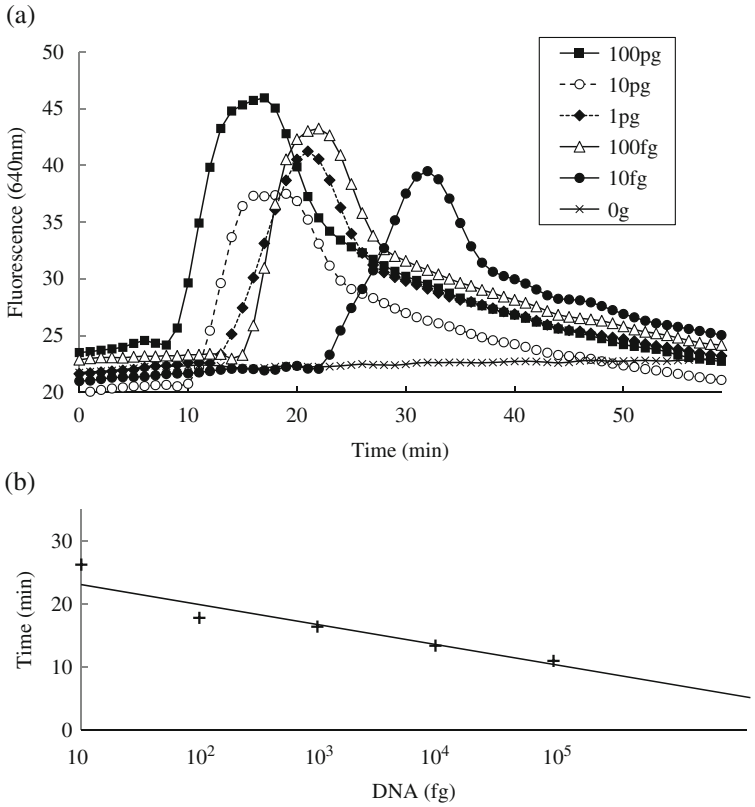

Fig. 9.3 Quantification of *Leishmania donovani* DNA using LAMP with SYBR Green detected by LineGene. **a** Real-time monitoring of LAMP reaction using LineGene. DNA of *L. donovani* were serially diluted from 100 pg to 10 fg and amplified using LAMP. The fluorescence intensity of DNA binding SYBR Green was monitored at every 1 min. **b** Standard curve for *L. donovani* LAMP assay generated from amplification plots between the ten-fold serially diluted *L. donovani* DNA

9.4 Combination of the Urine ELISA and LAMP

For confirmation of VL, LAMP is a simple and sensitive method, but it necessitates the use of blood samples and procedures to separate DNA from them. Urine ELISA to detect antibodies in urine samples is a simple and sensitive tool, but it has the disadvantage that past and present infections are indistinguishable, although a careful history and physical examination by an experienced clinician might aid the diagnosis. Urine sample collections have many advantages compared to blood sampling: they are non-invasive, with little chance of accidental infection, and they are expected to gain improved compliance of residents, especially those with no symptoms. In addition, urine collection is done easily. It requires no medical staff for its collection. Therefore, we propose a strategy for finding endemic foci of VL and asymptomatic carriers with a combination of mass screening using urine ELISA followed by LAMP for the confirmation of VL. It is also useful for fixed-point monitoring of VL. This strategy is expected to contribute to the control of VL.

9.5 Conclusion

We propose a strategy for the control of VL. It consists of two steps: mass screening to find the antibody positives, with subsequent parasite DNA detection with LAMP to confirm the infection. Furthermore, LAMP is useful to monitor the effectiveness of treatments and, therefore, to monitor the drug resistance of the parasite.

Acknowledgements Research underlying this chapter, conducted by the authors, was partially supported by Special Coordination Funds for Promoting Science and Technology (Grant no. 1200015), MEXT, Japan, and also by a Grant-in-Aid for Scientific Research (B) No. 18406013 from the Japan Society for the Promotion of Science.

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Part IV
PKDL and Its Implications in Eliminating Kala-Azar

Chapter 10
Polymorphism of Leishmaniasis Caused by *Leishmania donovani* Sensu Lato in Asia

Yoshitsugu Matsumoto, Chizu Sanjoba, Masahito Asada, Yasutaka Osada, and Yasunobu Matsumoto

Abstract Visceral leishmaniasis (VL), also called kala-azar (KA), is the most severe form of leishmaniasis and often fatal if not treated. The causative species of VL are members of *Leishmania donovani* sensu lato (s.l.), which includes *L. donovani*, *L. infantum*, *L. archibaldi*, and *L. chagasi*. Its principal victims are children and young adults. Control of KA is urgently needed worldwide. However, due to the diversity of the epidemiological features in each endemic focus, it has not been possible to devise a universal control strategy applicable to all foci. In order to construct a framework of epidemiological, pathological, and taxonomic understanding, and explain how *Leishmania* species develop different clinical and epidemiological features, further studies on the pathogenesis of different forms of KA and comparative studies of the parasites from different foci are clearly necessary. This chapter summarizes the recent advances in understanding the polymorphism of leishmaniasis as caused by *Leishmania donovani* s.l. in Asia.

Keywords Cutaneous leishmaniasis · Immunopathology · Kala-azar · *Leishmania donovani* · Post-kala-azar dermal leishmaniasis · Visceral leishmaniasis

Abbreviations

CL	Cutaneous leishmaniasis
ELISA	Enzyme-linked immunosorbent assay
KA	Kala-azar
NNN	Novy-MacNeal-Nicolle
PCR	Polymerase chain reaction
PKDL	Post-kala-azar dermal leishmaniasis
Sb	Sodium stibogluconate
s.l.	*sensu lato* (in a broad sense)
s.s.	*sensu stricto* (in a strict sense)
VL	Visceral leishmaniasis

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_10,

10.1 Polymorphic Leishmaniasis

Leishmaniasis is a vector-borne disease caused by parasitic protozoa of the genus *Leishmania,* which belongs to a group of unicellular protozoa known as hemoflagellates or trypanosomatid flagellates (Kinetoplatidae). This disease is widespread in the temperate, subtropical, and tropical regions of every continent except Antarctica. The parasites are digenetic with two morphological forms: promastigotes in the sandflies and amastigotes in vertebrate hosts. The promastigotes, motile flagellated forms, are transmitted by blood-sucking sandflies. When the female sandflies ingest blood meals, the parasites are delivered into the skin of vertebrate hosts and infect macrophages in which they then differentiate into amastigote form and proliferate intracellularly. Clinical symptoms range from simple skin lesions to mucocutaneous lesions, and death can result when visceralization occurs. Simple cutaneous leishmaniasis (CL) is characterized by the development of skin lesions, which may evolve from small erythematic or nodular indurations to crater-like ulcers with dry or wet centers, but all are self-limiting and self-healing in the course of several months. Diffuse CL is characterized by the formation of multiple skin lesions or ulcers due to the dissemination of parasites or multi-infection. Mucocutaneous leishmaniasis is complicated by the spread of infection from the skin to the nasopharyngeal cavity, often resulting in facial disfigurement. Visceral leishmaniasis (VL), also called kala-azar (KA), manifests as a chronic disorder affecting hematologic and hepatosplenic functions. KA is clinically characterized by fever, weight loss, anemia, and hepatosplenomegaly, and has a high mortality rate in untreated cases.

This polymorphism was for a long time thought to be largely a function of the parasites involved. Although more than 20 species have been described as pathogenic to humans, dozens of other species of the genus *Leishmania*, which include, e.g., parasites of lizards, are nonpathogenic to humans. Pathogenic *Leishmania* species have been differentiated by serodeme, zymodeme, and schizodeme analyses, which are based on the antigenicity, the electrophoretic mobility of their isoenzymes, and the endonuclease restriction fragments of their mitochondrial or kinetoplast DNA, respectively. However, genotype does not always correlate with current taxonomy or clinical manifestations. Phenotyping by multilocus enzyme electrophoresis, the most widely used method for classification of *Leishmania* parasites, does not always correlate with genotyping, either. The differentiation of *Leishmania* species from various geographic regions has proven difficult not only in terms of clinical criteria, but also with regard to the reference standard techniques for *Leishmania* typing.

VL, the most severe form of leishmaniasis, is caused by members of *Leishmania donovani* sensu lato (s.l.), which includes *L. donovani, L. infantum, L. archibaldi,* and *L. chagasi.* Recently, *L. chagasi* has been considered synonymous with *L. infantum*, and whether *L. archibaldi* is indeed an independent species has been questioned [1, 2]. On the basis of recent molecular studies, *L. infantum* and *L. donovani* have been widely accepted as independent species. According to this classification, *L. infantum* predominates in Europe, North Africa, and South and Central America, while *L. donovani* predominates in East Africa and India. In

China, both species are distributed [3]. So far, there are no reliable discriminative markers that can characterize their diversity in terms of geographical and epidemiological features. Rather, available data have indicated that *Leishmania* speciation is more complicated than previously thought.

10.2 Pathogenesis of Leishmaniasis

In all cases of mammalian leishmaniasis, macrophages are the exclusive host cells in which amastigotes can proliferate. The *Leishmania* parasites have common pathways to achieve the infection of their host, leading ultimately to their intracellular residence in macrophages. Lipophosphoglycan and gp63 are well-known *Leishmania* molecules involved in these pathways. Other surface molecules that bind to antibodies are also involved through endocytosis using Fc receptors.

In CL, the infected macrophages together with uninfected macrophages localize in the dermis or subcutaneous tissues, forming erythematic or nodular lesions. Crater-like ulcers are formed by the accumulation of infected macrophages at the rim of the ulcer with a necrotic center. Recently, we reported that macrophages expressing MRP8 and MRP14 predominate among the accumulated macrophages and also possibly serve as host cells [4, 5]. MRP8- and MRP14-positive macrophages are inflammatory cells and might play an important role in the pathogenesis of CL. The migration of amastigotes, or infected macrophages harboring amastigotes, to visceral organs culminates in VL. The mechanisms by which amastigotes or infected macrophages migrate to visceral organs have remained unexplained. In VL, the infected macrophages are localized in the spleen, liver, lymph nodes, and bone marrow, but infected cells must also be present in the skin or bloodstream to serve as a source of parasites for transmission to the vector. In mice experimentally infected with *L. major*, a causative species for human CL, infected macrophages are easily found in visceral organs including spleen, liver, and lung once the disease has progressed. Thus, the localization of amastigotes is not determined only by the characteristics of the parasites, but also by factors in the host. It has recently been demonstrated that macrophages are not a uniform cell population but rather show diversity that is expressed by surface markers or cytokine profiles. Identification of the particular macrophage populations that serve as host cells for each form of leishmaniasis may lead to better understanding of the pathogenesis of leishmaniasis.

There is a consensus of opinion that the virulence of leishmaniasis, as manifested in different clinical symptoms, is a function of host–parasite interactions. The pathology is due not only to the destruction of the host macrophages by the proliferation of amastigotes, but above all to the immunopathological response of the host to the parasite antigens. The interaction of the immune cells with the parasite may possibly lead to the production of cytokines, which modulate the host's immune reaction, resulting either in healing or the exacerbation of the disease. The

severity of the immunopathology in leishmaniasis can manifest as a spectrum of host immune responses, ranging from anergy to hyperactivity.

10.3 KA in Asia

Many aspects of KA in South Asia are described in other chapters in this book, so it will be mentioned here only briefly. About 500,000 cases of VL occur annually in the eastern and northern parts of Africa, the Mediterranean, the Middle East, Central Asian countries, Pakistan, India, Bangladesh, and China. A small focus has recently been reported in Bhutan. More than 90% of the world's cases of VL occur in India, Bangladesh, Nepal, and Sudan. In endemic areas, children and young adults are its principal victims.

From an epidemiological point of view, two types of KA are distinguished: anthroponotic and anthropozoonotic. The Indian type of KA, caused by *L. donovani* sensu stricto (s.s.), is believed to be anthroponotic. On the other hand, the KA caused by *L. infantum* found in the Mediterranean, where VL occurs almost exclusively in children (infantile VL), is anthropozoonotic with a dog reservoir. Both types of KA have largely discrete geographical distributions. However, several different types of VL have recently been observed in China [3]. The anthroponotic type of KA (caused by *L. donovani* s.s.) is seen in old oases in the Xinjiang Uygur Autonomous Region of China. The zoonotic type is further divided into mountainous and desert types. Mountainous type KA (caused by *L. donovani*) is seen in the western regions of China, including Gansu, Sichuan, and Shanxi provinces, where dogs have a high rate of infection and are considered the main reservoir. The patients are mostly children under 10 years of age. The fact that the elimination of dogs in certain areas has markedly reduced the number of cases supports the belief that dogs are major reservoir animals for KA in this region. The desert type of KA (caused by *L. infantum*), infantile leishmaniasis, is endemic in the northwest desert region, including Xinjiang and Inner Mongolia. These regions were uncultivated desert for a long time and only recently were populated by immigrants. Wild animals are presumably the source of infection. Interestingly, both types of KA are distributed in geographically defined areas in Xinjian [3, 6]. An outbreak of desert-type zoonotic leishmaniasis caused by *L. infantum* recently occurred in Xinjiang [6].

10.4 Post-kala-azar Dermal Leishmaniasis in Asia

Post-kala-azar dermal leishmaniasis (PKDL) is a well-known complication associated with KA, and is mainly seen on the Indian subcontinent and in Sudan [7]. PKDL is characterized by the development of skin lesions at variable times after the resolution of VL. In India, PKDL manifests in 5–15% of VL cases after months or several years of remission from infection, while in Sudan, it develops within weeks or months in 50–60% of cured VL cases. The most common PKDL skin lesions are multiple hypopigmented macules with irregular margins

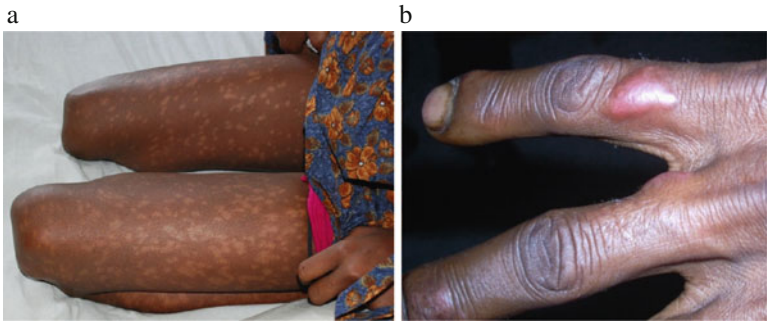

Fig. 10.1 Clinical manifestations in PKDL patients in Bangladesh. **a** macular lesion on the thigh; **b** papular lesion on the finger from which parasites were isolated in NNN culture

(Fig. 10.1a). Others are blister-like papules or nodules (Fig. 10.1b), but these are rare (<5% in Bangladesh). Some patients develop such skin lesions in combination. Histopathological sections of these macules show that the tissue of the skin is well organized with slight inflammatory infiltration. Nodular-type lesions show compact epithelioid granulomas (Fig. 10.2).

In Bangladesh, PKDL has been diagnosed by the development of skin lesions, a past history of KA, and a positive rK39 dipstick test. Although PKDL patients do not manifest severe symptoms other than the skin lesions, they generally receive three courses of treatment with sodium stibogluconate (Sb) since they are considered the only reservoir for KA on the Indian subcontinent.

PKDL was first described by Brahmchari in 1922 in a cured KA patient who suffered skin eruptions and plaques. The diagnosis was confirmed by the demonstration of amastigotes in a slit skin smear [8]. Amastigotes, however, were scant or absent in the macular lesions. In India, *Leishmania* parasites are detected in around 80% of the skin lesions of PKDL patients by polymerase chain reaction

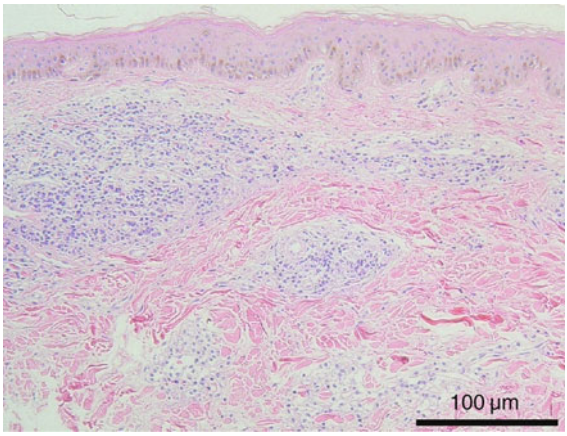

Fig. 10.2 Hematoxylin and eosin-stained section of skin from nodular lesion of PKDL patient. No parasites are observed

(PCR) [9]. However, parasites are detected in only 36% of macular lesions. We have successfully established a *Leishmania* strain taken from a papular lesion on the skin of a PKDL patient (MHOM/BD/08/SB6), but failed to isolate any parasite in Novy-MacNeal-Nicolle (NNN) culture from macular lesions in the same patient. It seems that the parasite burden in the skin lesions of PKDL patients differs based on the type of manifestation. Since *Leishmania* parasites were detected from *P. argentipes* experimentally fed from the PKDL patients [10], this report strongly supports the importance of PKDL patients as a reservoir for new transmission of KA. In the experiment, all PKDL patients showed nodular or noduloulcerative skin lesions and not macular lesions.

All these observations indicate that skin lesions in PKDL are not directly caused by the proliferation of *Leishmania*. Instead, it is suggested that host immunity is involved in the formation of these skin lesions. A recent study of local immunity in the skin lesions of PKDL patients suggests that cytokines such as interferon-gamma, tumor necrosis factor-alpha, and interleukin-6 play important roles in the development of skin lesions [11]. Thus, a detailed analysis of the immune reaction of PKDL patients will be helpful in understanding the pathogenesis of PKDL.

The seroreactivity of the PKDL patients was examined by enzyme-linked immunosorbent assay (ELISA) using *L. donovani* whole antigen and rK39 antigen, both resulting in reactions comparable to those of active KA patients. Recombinant K39 peptide (rK39) contains tandem repeats of a 39-aa peptide in an intracellular protein, kinesin, which is expressed stage-specifically in amastigotes of all visceralizing species. In patients with VL, this antigen elicits the production of antibodies to extremely high titers [12]. Seroreactivity to rK39 is believed to correlate with the presence of the parasites in visceral organs. Although PKDL patients do not show any hepatosplenomegaly, parasites might remain in the visceral organs even after recovery from KA.

There is no clear evidence that shows a difference between the causative parasites of KA and PKDL, although several studies have tried to resolve this issue [13, 14]. We have also performed DNA sequence analysis to find differences at the molecular level between isolates from PKDL patients and isolates from KA patients in Bangladesh. The results so far, obtained from the sequences of the mitochondrial peroxiredoxin gene and the cysteine protease B gene, have shown that the sequences of PKDL isolates were identical to those from the KA patients.

At the same time, the involvement of parasite factors in the pathogenesis of PKDL has remained unclear. The most widely believed explanation is that PKDL is associated with a relapse of the visceral form of the disease after an inadequate treatment for KA has failed to kill the parasite in the visceral organs [15]. The number of PKDL patients in India is said to be decreasing now that amphotericin B is being used for treatment [16]. This implies that adequate treatment of KA will be effective to minimize the occurrence of PKDL. However, there is no explanation for how and why the surviving parasites migrate from the visceral organs to the skin after inadequate treatment. One explanation might be reinfection in partly immunized individuals.

PKDL patients show no constitutional symptoms in the majority of cases. Skin lesions develop around 1 year after the clinical cure of KA. Most PKDL patients show only macular lesions; papular or nodular lesions are rare. However, these patients receive three courses of Sb treatment, and sometimes the adverse effects of the Sb include fatality [17]. It is likely that PKDL patients with papular lesions serve as a reservoir for KA, but there is no evidence suggesting that PKDL patients with only macular lesions are an important reservoir for KA. Further differential assessments of the parasite burden in each type of skin lesion are clearly needed.

10.5 CL Caused by *L. donovani*

Several sporadic cases of CL caused by *L infantum* have been reported in Italy [18] and France [19]. CL resulting from *L. donovani* s.s. without visceral symptoms or history of VL treatment is considered rare [20], although PKDL is known to be a common complication, as described above.

Recent studies have demonstrated that human indigenous CL caused by the parasites belonging to *L. donovani* s.l. is distributed throughout the island of Sri Lanka. More than 1,000 cases have been reported so far. Both CL and VL had not been known to occur in Sri Lanka until 1992, although there were a few imported cases [21]. The patients showed papule, nodule, and blister skin lesions without visceral symptoms or history of VL treatment.

In our study of 25 Sri Lankan patients with CL confirmed by detection of amastigotes on Giemsa stained smears (20 positives) or in NNN cultures (21 positives) from skin aspirates, the skin lesions of patients most commonly presented on exposed areas of the body, such as the face, arms, and legs. The lesions were mostly papules or blister-like nodules. Only three patients presented ulcers. The number of lesions observed in a single patient was low (n = 1–3). Only one patient had more than three lesions. The skin lesions were small and varied from 5 to 20 mm in diameter. The lesions of these patients lasted three to 36 months (Fig. 10.3 a, b). None of the individuals with cutaneous lesions had experienced fever or anemia either before

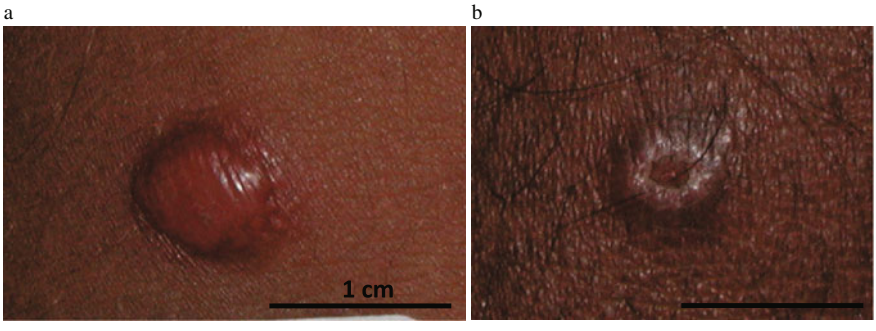

Fig. 10.3 Clinical manifestation of skin lesions in Sri Lankan CL patients. **a** papular lesion of 4 months' duration on the abdomen; **b** nodular-like lesion of 8 months' duration on the arm

or after the appearance of the skin lesions, and they had no history of other diseases, including immunosuppression. Although three patients showed lymphadenopathy, three patients showed slight hepatomegaly, and one patient showed splenomegaly, other symptoms typical for VL were not apparent in these patients, and there was no evidence that these symptoms had been caused by the leishmanial infection. None of the patients had a history of known or suspected VL in the past.

To identify pathogenic species, the nucleotide sequences of a region of the actin-encoding gene, which is highly conserved among eukaryotes, and those of the mini-exon gene, which is unique in the genus *Leishmania*, were analyzed using the isolated parasites. The comparison of the nucleotide sequences showed the highest homology with that of *L. donovani* s.s., but those of Sri Lankan isolates were not identical with any reported species belonging to *L. donovani* s. l. These results demonstrated that the dermotropic *Leishmania* parasite in Sri Lanka belongs to *L. donovani* s.l. Enzyme analysis also showed that Sri Lankan *Leishmania* isolates were characterized as *L. donovani* zymodeme MON-37 [22]. Zymodeme MON-37 has been previously reported in cases of human VL in India and southern Ethiopia [23, 24], but the isolation of *L. donovani* zymodeme MON-37 from human cutaneous cases has never before been reported.

ELISA using the rK39 antigen and crude antigen prepared from *L. donovani* showed that the optical density values of plasma from Sri Lankan CL patients were very low (0.30 ± 0.2; $n = 11$) compared with those of KA patients in India and China (>3.00). In addition, the rK39 dipstick test for VL (Kalazar Detect; InBios International, Seattle, WA, USA), an antibody detection assay to rK39 antigen and highly sensitive for VL caused by members of *L. donovani* s.l., showed all patients to be negative. Patients with CL or mucocutaneous leishmaniasis often have few *Leishmania* antibodies, because of the localized character of the disease, and thus serological tests are usually negative [12]. Serological examination using rK39 antigen did not show any sign of visceral localization of amastigotes in the Sri Lankan patients.

The question arises whether this emergence of the disease is due to the recent introduction of the parasite from other endemic areas such as India, or whether we are merely seeing the recognition of an existing disease, or else the emergence of a new type of leishmaniasis due to mutated *L. donovani*. The presence of dermatropic *L. donovani* should be noted, although host factors may play a role.

10.6 Working Toward the Control of VL

Control strategies for leishmaniasis have focused on four aspects: reservoir control, vector control, and control by chemotherapy and vaccination. It is widely recognized that blocking the transmission cycle is key in the control of leishmaniasis. However, due to the diversity of the reservoirs, vectors, and parasites, leading to different epidemiological features, it has not been possible to devise a universal control strategy applicable to all foci of the leishmaniases.

For a long time, PKDL patients had been considered the most important reservoirs in the transmission cycle in South Asia. Since PCR successfully detects parasite DNA in samples taken from VL patients and some subclinical individuals, these may be the source of the parasites. There is increasing evidence indicating that subclinical infection, including latent or dormant infection, is more common than previously thought.

In some foci of anthropozoonotic VL, dogs, foxes, jackals, wolves, and rodents have been recognized as reservoirs, and the disease in humans is limited to areas inhabited by these reservoir animals. The VL caused by *L. donovani* s.s. in India, Nepal, and Bangladesh has been thought to be anthroponotic; that is, transmission occurs mainly between humans and sandflies without the involvement of reservoir animals. This does not exclude the possibility of the involvement of infected animals. Recently, in Nepal, the involvement of domestic animals such as cows, buffaloes, and goats has been suggested [25]. The identification of the real reservoir(s) serving as the most effective source(s) of transmission to humans is urgently needed for the control of the disease, and this identification should be achieved as soon as possible.

The endemism of human leishmaniasis caused by dermatropic *L. donovani* s.l. in Sri Lanka is unique in the world. This finding emphasizes the unreliability of the identification of *Leishmania* species solely on the basis of clinical presentation. It is expected that the comparison of the PKDL isolates with the isolates from Sri Lankan CL will provide a better understanding of the tropism of *L. donovani*.

Acknowledgments The authors would like to thank Dr. Yoshihisa Hashiguchi (Kochi University) for providing *L. donovani* strains from KA patients, and Dr. Akram Hossain and Dr. A.K.M. Shamsuzzaman (Mymensingh Medical College) for helping research on patients in Bangladesh. The authors also wish to thank Dr. R.P.V. Jayanthe Rajyapakse and Dr. R.R.M.L.R. Siyambalagoda (University of Peradeniya) for supporting our Sri Lanka field research. This study was supported by a Grant-in-Aid for Scientific Research (A) (19256001) from the Ministry of Education, Science, Sports, and Culture in Japan.

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Chapter 11
Post-kala-azar Dermal Leishmaniasis: Facing the Challenge of Eliminating Kala-Azar from South Asia

Philippe Desjeux and V. Ramesh

Abstract Post-kala-azar dermal leishmaniasis (PKDL) is a cutaneous complication of visceral leishmaniasis (VL) which usually appears after treatment of a VL episode. The incidence of PKDL varies across countries and no up-to-date global estimate is available. Parasitological diagnostic tests show either low sensitivity or are difficult to decentralize in the field (e.g., polymerase chain reaction). Available treatments are long, costly, and frequently toxic. It is believed that PKDL has a multi-factorial and complex origin. It is widely accepted that persons with PKDL harbor *Leishmania* parasites in the skin and, therefore, act as reservoirs of infection in VL transmission, especially during interepidemic periods. PKDL poses a serious threat to the success of the VL elimination program in South Asia, and requires an immediate and focused strategy from the health authorities in charge of the national programs in India, Nepal, and Bangladesh. Control and research efforts are urgently needed to improve PKDL surveillance and case management in order to reduce delays in diagnosis and treatment and, hence, reduce morbidity and the risk of transmission.

Keywords Bangladesh · India · Kala-azar · Nepal · Post-kala-azar dermal leishmaniasis · Visceral leishmaniasis elimination program · Visceral leishmaniasis

Abbreviations

ACD	Active case detection
BCC	Behavior change communication
ELISA	Enzyme-linked immunosorbent assay
HIV	Human immunodeficiency virus
IEC	Information, education, communication
IL	Interleukin
kDNA	Kinetoplastid DNA
LAMP	Loop-mediated isothermal amplification

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_11,

LDB	Leishman-donovan body
LST	Leishmanin skin test
PCR	Polymerase chain reaction
PKDL	Post-kala-azar dermal leishmaniasis
Sb5+	Pentavalent antimonials
VL	Visceral leishmaniasis

11.1 Clinical Features

The majority of reported post-kala-azar dermal leishmaniasis (PKDL) cases have been from the Indian subcontinent and East Africa, primarily Sudan. Indian PKDL has been reviewed [1], and patients were primarily from the eastern regions of India, as well as Nepal and Bangladesh. The polymorphic form of PKDL is the most frequently encountered: hypochromic macules, indurated erythematous plaques, papules, and nodules are seen in varying proportions. The typical form is that of lesions clustered around the nose and mouth, with discrete to no lesions on the rest of the face (Fig. 11.1). At times the lesions may be present on the sides of the cheeks and the ears, leading to the potential for confusion with leprosy. Over time, these lesions can coalesce and give rise to large tumor-like plaques which are well circumscribed. Disease progression is nearly always from the face to the rest of the body, including the feet. Initially, the disease manifests as a transient and recurrent photosensitive eruption that later presents with skin lesions. When lesions are localized, the face is most affected, with other areas remaining relatively unaffected. Sensitivity to sunlight is attributed to this preferential commencement of the disease, which may later spread to the rest of the body.

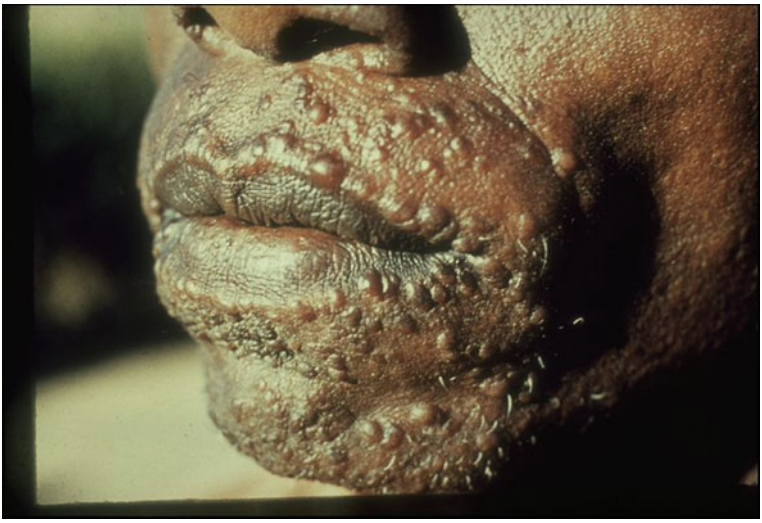

Fig. 11.1 Numerous typical papules around the mouth

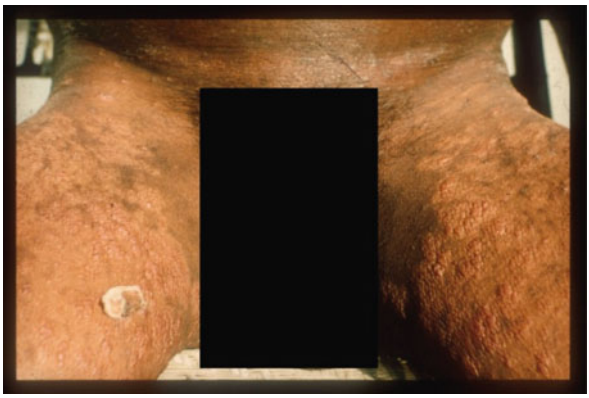

Fig. 11.2 Hypopigmented coalescent plaques with incipient nodules

Up to one-fifth of PKDL patients display mucosal lesions affecting the glans penis and oral cavity. If the outer lips are taken into account, this figure rises considerably, since lesions are frequently present in this area. Impact on the mucosa without involvement of the skin is extremely rare and has been observed only occasionally in the highly endemic area of Bihar [2]. Similarly, ocular lesions on the cornea and sclera were rarely seen in the distant past [3]. The unusual forms of the disease are those that are often missed and unsuspected. Of these, the macular form of PKDL is the most important. It is more likely to be mistaken for vitiligo rather than leprosy since the degree of pigment loss is much greater than that seen in leprosy. Macular PKDL accounts for around 10% of PKDL patients, and its distribution can be localized, generalized, or extensive; such lesions lack a photosensitive evolution and at times the face may be minimally or not affected [4, 5]. Remarkably, hypopigmentation may affect the entire skin surface, leaving only a few areas of normal or hyperpigmented skin, particularly at the flexures (Fig. 11.2). Others have also observed a similar incidence of macular PKDL in their studies [6]. The other presentation of PKDL is the fibroid variety with plaques on the dorsa of fingers and toes, with an appearance like knuckle pads.

African PKDL, mainly seen in Sudan, has been increasingly reported and has been reviewed by Musa et al. [7] and Zijlstra et al. [8] The causative species remains the same as in the Indian variety. In contrast to Indian PKDL, which occurs in 5–10% of those treated for kala-azar, Sudanese PKDL appears in as many as 50% of patients who have received therapy for kala-azar. The distribution of lesions in Sudan is similar to that of the Indian form but differs in the ulceration seen in severe cases. An interesting study in Sudan showed that the severity of facial involvement was related to ultraviolet B radiation in sunlight, which appears to modify the immune system, promoting lesion development [9]. Mucosal involvement in African cases also differs from that of Indian PKDL in that lesions have been more frequently noted in the eyes or restricted to ocular mucosa [10], giving rise to a number of terms such as post-kala-azar ocular or mucosal leishmaniasis. These could well be subsumed under the term PKDL which also covers mucosal lesions, avoiding further confusion.

Patients with human immunodeficiency virus (HIV) receiving anti-retroviral treatment have experienced PKDL as part of the immune reconstitution inflammatory syndrome. Such patients who have been observed mainly in Europe and South America have been briefly reviewed by Antinori et al. [11] The species in these HIV-positive patients is *L. infantum* rather than the *L. donovani* common in India and Sudan, and the clinical picture may be quite different from the classical forms described above. Hence, clinical suspicion and appropriate investigations hold the key to diagnosis.

11.2 Epidemiology

Information on incidence and prevalence of PKDL is fragmentary, and few studies are available. Recently, the first population-based study of PKDL incidence was completed in Bangladesh [12]. It combined a house-to-house survey (2007–2008) and a retrospective study that included 22,699 people from 4,553 households. In total, 813 cases of kala-azar were detected with 79 PKDL cases (9.7%) among them. Among the 813 VL cases, 783 had been treated with pentavalent antimonials (Sb5+) and 30 with miltefosine. It is noteworthy that 8 of the 79 PKDL cases (10.1%) had no previous history of VL. The median delay between the VL episode and PKDL was 22 months. More specifically, 20% of PKDL cases appeared within 6 months, 40% between 6 and 24 months, and 40% after 24 months. Uncommon events were also reported such as PKDL occurring concomitantly with VL or a VL relapse and spontaneous cure. It is particularly relevant to note that PKDL incidence rose 21-fold in Bangladesh from 2002 to 2007 (from one case per 10,000 population to 21 cases per 10,000 population), and is still rising even though the VL incidence decreased from 25 cases to 15 per 10,000 population during the same time period. PKDL appears as an echo of the 2004–2006 VL epidemic [12].

In India, PKDL appears in 5–10% of treated VL cases and occurs within one to 3 years after treatment, as reported by Ramesh and Mukherjee in 1995 [1]. Some authors, based on a facility-based retrospective data analysis, have made a link between the decline in PKDL incidence and the introduction of amphotericin B and, more recently, miltefosine [13, 14].

In Nepal, information on PKDL is fragmentary and usually obtained through facility-based passive surveillance. In a study of 50 cases between 1998 and 2005, 80% had a past history of kala-azar, and most patients had polymorphic lesions (macules, papules, and nodules) [15]. Seventy-eight percent were parasitologically confirmed by slit skin smears.

11.3 Diagnosis

The diagnosis of PKDL is initially based on the typical clinical features (macules, papules, and nodules). Clinically the most frequent differential diagnoses are vitiligo, pytiriasis versicolor, viral warts, and mycosis fungoid. However, two

diseases deserve special mention: neurofibromatosis and leprosy, which can be differentiated by the ulnar nerve thickness and the loss of skin sensitivity on depigmented patches. The diagnosis of PKDL is usually supported by the epidemiological background: most patients report a treated VL episode, usually in a focus of anthroponotic transmission. The laboratory methods of diagnosis are immunological and parasitological. Serological methods used for kala-azar diagnosis, such as the direct agglutination test, anti-rk39 antibody detection using immunochromatographic strips, and enzyme-linked immunosorbent assay (ELISA) based on recombinant antigens, have been applied successfully to PKDL diagnosis [16, 17]. It should be kept in mind that a positive antibody test may be the result of the previous VL episode rather than current PKDL. Antigen detection in tissues, blood, and urine is also of some help. However, only parasitological methods are confirmatory. The slit skin smear and culture are the standard methods, but their sensitivity is low (up to 54% of cultures).

In all types of PKDL, the finding of organisms is a bedside aid to diagnosis. The nodular type of lesion is most likely to show these amastigotes, termed Leishman-Donovan bodies (LDBs). The likelihood of finding LDBs diminishes in less indurated types of skin lesions and is lowest in the macular variety.

Molecular methods based on nucleic acid detection, using gene amplification techniques such as polymerase chain reaction (PCR), provide a reliable means of species-specific diagnosis of the disease. These methods are more sensitive than immunohistochemical or serological methods. Gene amplification is carried out by targeting multicopy sequences, such as ribosomal RNA genes, kinetoplastid DNA (kDNA), miniexon-derived RNA genes, or genomic repeats [16, 17]. A kDNA-based PCR assay developed in India detected the parasite in 45 of 48 PKDL patients with 93.8% sensitivity [18]. Different PCR assays are now available, including real-time PCR, nested PCR, and loop-mediated isothermal amplification (LAMP) PCR. They can detect *Leishmania* parasites in blood or on slit skin. LAMP PCR has several advantages: an easier-to-run kit is available, no sophisticated equipment is needed, the required temperature for DNA amplification is lower (62–65°C), and the test duration is shorter (1 hour only). In addition, the reading is easy (turbidity). It is as sensitive as nested PCR, which is claimed to be more sensitive than traditional PCR. LAMP PCR is also claimed to be a good test of cure, as it becomes negative 2 weeks after treatment. However, it is difficult to decentralize the test further than the district level. At the level of peripheral microscopy laboratories, severe limitations remain: (1) lack of skilled and motivated staff; (2) risk of DNA contamination; (3) difficulty of interrupting LAMP procedure to cope with other tasks; and (4) need for a continuous power supply [19]. Based on experiences in Bangladesh with VL patients, it was noted that the collection of blood on filter paper does not yield good results. One milliliter of blood is needed and presents constraints of transport. While a significant potential for further simplification of the method exists, it requires further research [20].

The main limitation is that most of these tests are unavailable or not performed in local institutions. The diagnosis of PKDL when LDBs are not demonstrable often rests on the clinical picture and the response to anti-leishmanial therapy. In the case

of PKDL in Sudan, the disease frequently occurs during or soon after the treatment of kala-azar, making the diagnosis relatively easy and with less likelihood of confusion with other dermatoses, as in the case of Indian PKDL.

11.4 Histopathology

Histopathological examination in PKDL shows several changes that often occur in combination. The epidermal changes include hyperkeratosis, parakeratosis, follicular plugging, focal acanthosis or, rarely, atrophy. Dermis shows mixed inflammatory infiltrate consisting of histiocytes, lymphocytes, and plasma cells.

In African cases, lymphocytes are the predominant cells, followed by histiocytes and some plasma cells. Epithelioid cell granulomas were seen in about 20% of cases and scattered epithelioid cells in about half [8]. Compact granulomas were seen more commonly in nodules than in papules [21]. Neuritis, an unusual finding, has been reported in four of 15 cases and has to be differentiated from leprosy [8]. Parasites are seen in 17–20% of cases on routinely stained sections; this sensitivity increases to 88% when a specific monoclonal antibody was used to stain *L. donovani* [22].

In Indian cases, lymphocytes and plasma cells are the predominant cells, with only scattered macrophages [23]. Epithelioid cell granulomas are rare. A low parasite detection rate was also seen in the predominantly nodular type of Indian PKDL [24]. In another study, the parasite detection rate increased from 50% using a hematoxylin and eosin stain to 80% when immunohistochemical staining for detection of *Leishmania* antigen was performed [25]. In our recent observations on 52 PKDL cases from India [26], morphological findings showed that the various patterns of dermal inflammatory infiltrate observed were diffuse dermal infiltrate perivascular alone, perivascular in combination with perifollicular and/or perieccrine, and rarely a curvilinear leprosy-like pattern. LDBs were seen in less than half the cases, and were greater in number and more easily appreciable in mucosal biopsies such as the inner lip or tongue.

11.5 Treatment

Treatments for PKDL are the same that are used for kala-azar, namely Sb5+, amphotericin B and miltefosine, but the duration of treatment is much longer [1, 27, 28]. Table 11.1 shows that the duration varies from two to four times that for kala-azar. Some of the unusual observations which need to be explained are the sensitivity of the parasite to the drug, its residence in the dermal macrophages, and the quantity of drug reaching the infected cells to achieve killing of the parasites. Some of the Sudanese forms of PKDL undergo spontaneous resolution but, for those that persist, the treatment varies, and may take up to 3 months or even longer [8]. Since the toxicity of sodium stibogluconate, the only choice for many clinicians, makes it extremely difficult for the patient to take until cured, most of the patients fail to

Table 11.1 Drugs used in the treatment of Indian post-kala-azar dermal leishmaniasis

Name of drug	Duration of treatment (months)		Mode of administration and dose	Drug delivery	Total cost of therapy in PKDL[a]	Remarks
	Kala-azar	PKDL				
Sodium stibogluconate	1	≥ 4	Intramuscular or intravenous 20 mg/kg/day up to 1 g/day	Inpatient or outpatient	$175	Cumbersome, but often only choice as compared to others
Amphotericin B (non-liposomal)[b]	0.5[c]	2	Intravenous 1 mg/kg/day up to a total of 3 g	Inpatient	$190	Impractical for routine use
Miltefosine	1	2–3	Oral 50 mg two or three times daily	Outpatient	$437	Too expensive and not yet freely available

[a]One US $ = 49 Indian rupees.
[b]Liposomal variety being prohibitively expensive has not been routinely used.
[c]15 days.

complete the recommended therapy. An attempt was made to reduce the duration of therapy for Indian PKDL by combining the drug with other weaker leishmanicidal drugs such as ketoconazole, rifampicin or allopurinol, and an immunotherapeutic agent, without much success [29, 30].

Currently available treatments for PKDL are of long duration, costly, and frequently toxic. Pentavalent antimonials are no longer recommended for PKDL in India because of the high level of resistance. However, in Bangladesh they remain the first-line drug. The usual treatment regimen is done over a 7-month period with a total of 120 injections at 20 mg/kg per day, with each month of antimonial use (n = 4) followed by an interval month of no antimonial use (n = 3). Cardio-toxicity is common. In India, amphotericin B is administered intravenously at 1 mg/kg every other day for two or three courses of 20 days with 15-day intervals. Its use is limited by the high cost, the need for hospitalization, and the frequent side effects. Miltefosine, an oral drug, is the most preferred treatment. It is currently under evaluation in India at 100 mg per day for 12 weeks. Results are promising, but there is no official recommendation yet [30].

Experience with liposomal amphotericin B (AmBisome) is still limited. In Sudan, 12 PKDL patients were treated with AmBisome at 2 mg/kg per day for 20 days after Sb5+ treatment failure; the cure rate was 83% (10 of 12). Macular forms were cured more slowly than papular ones. Safety was good with no reported side effects. Delay of cure was correlated with the age of lesions [31].

Immunochemotherapy is another option currently under evaluation. It is based on a combination of drug plus vaccine. In a recent clinical trial in Sudan, Sb5+ at 20 mg/kg per day for 40 days was combined with a first generation vaccine (alum-precipitated autoclaved *L. major* plus bacillus Calmette-Guérin). The combination was compared with the same Sb5+ regimen plus placebo. Thirty PKDL patients were treated, and safety and immunogenicity parameters were analyzed. Minimal local adverse events were reported. At 60 days, the cure rate was 87% for the vaccine plus Sb5+ versus 53% for the placebo plus Sb5+. At 90 days, there were no relapses for the vaccine arm and two relapses for the placebo arm. The leishmanin skin test (LST), which initially was negative, became positive after clinical healing or clinical improvement. The authors concluded that LST is a good surrogate marker of cure in persistent PKDL lesions [32]. Further research is needed to test the immunochemotherapy model with second-generation vaccines and different drugs (miltefosine, paromomycin, AmBisome), taking into account all parameters: not only safety, efficacy, and immunogenicity, but also cost and practicality.

11.6 Pathogenesis

PKDL seems to have a multi-factorial and complex origin combining host, parasite, drug, and perhaps genetic factors (linkage of the interferon-gamma receptor to PKDL and not to VL).

Host characteristics are mainly immunological and seem crucial for PKDL development. The host's role is suspected, based on: (1) the high incidence of PKDL in immunosuppressed people (e.g., transplant recipients and patients with

HIV/AIDS, tuberculosis, malaria, measles); (2) the efficacy of therapeutic vaccines with good and extended immunogenicity; and (3) the clinical healing associated with a conversion of LST from negative to positive [32].

Parasite characteristics are still under discussion. It is clear that PKDL incidence is much higher in *L. donovani* foci than in *L. infantum*. However, there is still an unanswered question of whether the higher incidence is related to the parasite and/or to the epidemiology. PKDL's higher incidence could be due to the anthroponotic (human-to-human) transmission in *L. donovani* foci compared to the zoonotic (animal-to-human) transmission of *L. infantum*. Molecular studies on clinical isolates from VL and PKDL cases have not shown major differences. To date, no strain has been associated with one single entity (VL or PKDL) in mixed endemic areas [33, 34].

Drug characteristics may also be important to PKDL development. However, some PKDL cases appear without any previous VL episode and, consequently, prior to any treatment. Much of the PKDL reported in the past has been observed after treatment of kala-azar with pentavalent antimonials. The introduction of newer drugs such as amphotericin B and miltefosine has led researchers to investigate the relationship between the efficacy of a given drug and the subsequent occurrence of PKDL. Having analyzed cases seen over 35 years from a high endemic area, the authors concluded that the incidence of PKDL declined after the introduction of amphotericin B for kala-azar in areas with high refractoriness to antimonials [13, 14]. It is now established that any drug can lead to PKDL but in very variable proportions (e.g., Sb5+ more frequently than amphotericin B). A few PKDL cases have already been reported after using miltefosine for VL treatment [35, 36]. PKDL could be the result of an immunological attack on *Leishmania* parasites which have survived in the skin despite chemotherapy. It is probably not the drug itself which leads to PKDL but the type of cellular immune response induced by the drug (cytokine profile) and the level of *Leishmania* parasite burden remaining in the body after treatment. Sb5+ has a specific influence on the immune response through its effects on cell signaling, cytokines, and immune complex induced levels of granulocyte macrophage colony stimulating factor [37]. Sb5+ and amphotericin B have contrasting effects on interleukin(IL)-10 and tumor growth factor beta in PKDL patients [38]. Only prospective clinical trials on VL patients treated with different drugs (Sb5+, amphotericin B, miltefosine, paromomycin, and AmBisome) and longitudinal follow-up will allow clear and final conclusions on the comparative rate of PKDL induced by each drug [13].

11.7 Challenges for VL Elimination

In South-East Asia, PKDL is now recognized as a classical complication of VL with important clinical and epidemiological implications. The main PKDL challenge is related to its strongly suspected role as a reservoir of infection. The hypothesis is based mainly on experimental laboratory work carried out in India, in 1992, which demonstrated the infectivity of PKDL cases. One hundred four laboratory-bred *P. argentipes* sandflies were fed by xenodiagnosis on nodular PKDL cases. Sixty

survived, and 32 (53%) were infected as evidenced by the presence of *Leishmania* promastigotes in the mid-gut. It was concluded that Indian nodular PKDL cases were highly infective for the sandfly *P. argentipes*, and it was extrapolated that PKDL cases can play a role as interepidemic reservoir [39]. A few years earlier, in 1988, another study concluded that the presence of as few as 0.5% durably infectious PKDL patients during an epidemic may cause VL to become endemic [40].

PKDL poses a second challenge: the perception of PKDL by the patients themselves. They consider PKDL as a chronic, cosmetic, non-fatal disease with a relatively severe stigma only if nodular. Hence, the motivation for early diagnosis and treatment is low. The delay between the onset of clinical manifestations and treatment is usually long, and leads to an increased risk of transmission to people living in the same household or close by. Sometimes the disease is even perceived as a favorable sign: "The disease has come out! The child will survive." In addition, as the treatment is long, costly, and frequently toxic, people are often reluctant to accept the treatment: "I will better die than to receive 120 injections!" Males and marriageable girls are usually more motivated to seek diagnosis and treatment.

11.8 Methods and Strategies to Control PKDL

In 2005, India, Nepal, and Bangladesh signed a Memorandum of Understanding to work regionally toward the elimination of VL by 2015. In order to increase the chances for success of the ongoing VL elimination program in India, Nepal, and Bangladesh, PKDL has to be addressed more urgently and more seriously. A series of control measures and research activities have to be undertaken quickly and simultaneously:

(I) *Control Measures*

- (I. 1) Improved surveillance combining passive surveillance and active case detection (ACD) to get a better estimate of PKDL incidence
- (I. 2) Early diagnosis and prompt treatment together with an improved referral system and mapping of PKDL cases
- (I. 3) Post VL-treatment longitudinal follow-up to detect the appearance of PKDL and an assessment of the drug-specific incidence rates
- (I. 4) Greater awareness among the communities, based on both IEC and BCC campaigns for improved acceptability of treatment
- (I. 5) Capacity building through institutional strengthening and training at all levels (especially health volunteers, lab technicians, and physicians)
- (I. 6) Distribution of long lasting nets to limit the infectivity of untreated PKDL cases

(II) *Research Activities*

- (II. 1) Identification of more effective, safe, short-course, affordable, accessible, and acceptable treatment regimens for PKDL

(II. 2) Identification of new treatment regimens (combinations) for VL to prevent the appearance of PKDL
(II. 3) Point-of-care diagnostic testing, adaptable to community-based ACD
(II. 4) Firm clinical and laboratory markers of PKDL that predict severity and parasitological cure after VL treatment (interleukins, especially IL-10 in skin biopsies, peripheral blood mononuclear cells, and in plasma)

Heightened political and financial commitments are urgently needed to implement the above recommendations. Actions have to be taken immediately, or else PKDL cases will remain a major impediment for the VL elimination programs especially, but not only, in Bangladesh.

These recommendations are not new. In India, in 1968, Sen Gupta quoted a recommendation made by Nappier in 1931: "PKDL doesn't appear to have received adequate attention of the medical profession and cases continue to go unrecognized and mistaken for leprosy and various skin diseases." [41] In 1989, in India, Alan and. Mukhopadhyay stated: "Therefore the PKDL cases are of great epidemiological importance and it should be stressed here that regular studies in the endemic areas should be undertaken and exact number of PKDL cases must be recorded particularly during the time when the prevalence of kala-azar is extremely low" [42]; and in 1992 Addy and Nandy made the following recommendation: "Therefore for the control of future outbreaks of kala-azar in India, adequate surveillance should be provided to diagnose and treat PKDL cases with a view to eliminate parasite source from the community." [39].

The importance of detecting and treating PKDL in kala-azar control programs is vital (Fig. 11.3). Programs must be implemented to effectively treat PKDL to bring about an end to transmission of VL. In an increasingly global world, it is important for practitioners of tropical medicine to be familiar with PKDL as it can be seen in

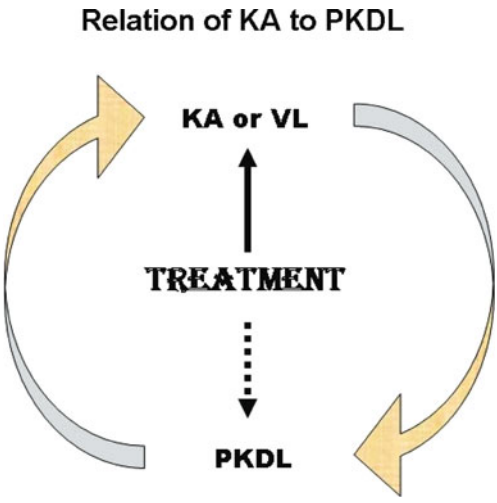

Fig. 11.3 Weak link (*broken line*) in kala-azar control

immigrants residing in the West several years after the episode of kala-azar [43] or in those from non-endemic countries such as Japan who return after a period of time in India [44].

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Part V
New Challenges Confronting Kala Azar Elimination Programme and Their Possible Solutions

Chapter 12
Climate Change and Kala-Azar

Moazzem Hossain, Eisei Noiri, and Kazuhiko Moji

Abstract Kala-azar or visceral leishmaniasis (VL) is a parasitic disease caused by *Leishmania donovani*. On the Indian subcontinent, it is transmitted by the sand fly, *Phlebotomus argentipes.* Kala-azar is found in about 88 tropical and sub-tropical countries with approximately 350 million people living in affected areas and at risk of infection. About 500,000 cases occur annually. More than 90% of the world's VL cases are in India, Bangladesh, Nepal, Sudan, and Brazil, affecting largely the socially marginalized and the poorest communities. In the South-East Asian Region, kala-azar occurs in India, Bangladesh, and Nepal with a small focus reported in Bhutan. In this region about 200 million people are "at risk." Fifty-two districts in India, 12 in Nepal, and 45 in Bangladesh are endemic. Most of the SEAR countries are vulnerable to the consequences of climate change. The ultimate effects of climate change are increased flooding, the breakdown of sanitation systems, increased salinity, more vector growth, and more water- and food-borne diseases, which ultimately impact human health. Environmental changes often modify the transmission patterns of vector-borne diseases. Increases in temperature due to climate change provide a better breeding environment for vectors, including the sand fly, in places where temperatures were previously below optimum, and so, a higher rate of human VL infection may result. Further research in this area is needed.

Keywords Climate change · Kala-azar · Leishmaniasis · South-East asian region

Abbreviations

GHG	Greenhouse gas
IPCC	Intergovernmental panel on climate change
NGO	Non-governmental organization
PKDL	Post-kala-azar dermal leishmaniasis
SEA	South-East Asia

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_12,

SEAR South-East Asia Region
SLR Sea-level rise
STH Soil-transmitted helminthiasis
VL Visceral leishmaniasis
WHO World health organization

12.1 Background

Kala-azar or visceral leishmaniasis (VL) is a complex parasitic disease caused by the trypanosomatid parasite *Leishmania donovani*. On the Indian sub-continent, it is transmitted by the sand fly, *Phlebotomus argentipes.* Leishmaniasis is found in about 88 countries. Approximately 350 million people live in these areas. About 500,000 cases occur annually, mostly affecting countries in the tropics and subtropics. More than 90% of the world's cases of VL are in India, Bangladesh, Nepal, Sudan, and Brazil, affecting largely the socially marginalized and the poorest communities. In the South-East Asian Region, kala-azar occurs in India, Bangladesh, and Nepal, with a small focus reported in Bhutan. In this region, about 200 million people are "at risk." Fifty-two districts in India, 12 in Nepal, and 45 in Bangladesh are endemic. In May 2005, the three countries signed a memorandum of understanding in Geneva during the World Health Assembly, committing to work toward the elimination of kala-azar from their respective countries by 2015. The objective of the elimination program is to reduce the incidence of kala-azar to less than one case of kala-azar and post-kala-azar dermal leishmaniasis (PKDL) per 10,000 population at the district level (in Nepal) or the subdistrict/upazila level (in Bangladesh and India) [1].

Human-induced changes in the global climate and the associated rise of sea levels are widely acknowledged among policy makers and scientists. The Intergovernmental Panel on Climate Change (IPCC) concluded, "The balance of evidence suggests a discernible human influence on global climate." The exact magnitude of the changes in the global climate is still uncertain and the subject of worldwide scientific studies. It is broadly recognized that Bangladesh is very vulnerable to these changes. Indeed, it has been argued internationally that Bangladesh, as a country, may suffer the most severe impacts of climate change [2].

During the twentieth century, the Earth's average surface temperature increased by approximately 0.6°C, and approximately two-thirds of that warming occurred after 1975 [3]. Climatologists forecast further warming, along with changes in precipitation and climate variability, during the coming century and beyond. Their forecasts are based on increasingly sophisticated global climate models applied to plausible future scenarios of global greenhouse gas (GHG) emissions. Changes in world climate are expected to influence the functioning of many ecosystems and their member species. Likewise, there will be an impact on human health. Scientists believe that most of the health effects of climate change will be adverse. Climatic changes over recent decades have probably already affected some health outcomes. Indeed, the World Health Organization (WHO) has estimated that climate change was responsible in 2000 for approximately 2.4% of worldwide diarrhea cases and

6% of malaria cases in some middle-income countries [4]. The first detectable changes in human health may well be alterations in the seasonality of certain infectious diseases, including vector-borne diseases such as malaria, filaria, leishmania, dengue, as well as cholera and other water- and food-borne infections that peak in the warmer months.

12.2 The Regional Impact of Climate Change

In recent years, the incidence of VL has increased in some countries of Tropical Asia. During 1987–1990, VL reached epidemic proportions in the Indian state of Bihar and spread rapidly to surrounding areas. In 1996, WHO estimated that about 110 million people were at risk of contracting VL. Major endemic foci are reported in border areas between India (the states of Bihar and West Bengal), Bangladesh, and Nepal. In Bangladesh, VL already has reached epidemic proportions; the most vulnerable populations are poor, rural cattle farmers. Reported cases appear to cluster close to flood-control embankments; there appears to be a significant risk that VL prevalence in some localities will increase as a result of flood-control and drainage projects [5]. The incidence of VL may also increase as the climate grows warmer (WG II, Section 18.3 [6, 7]).

12.3 Climate Change and the Spatio-Temporal Distributions of Leishmaniasis

Environmental change often modifies the transmission patterns of vector-borne diseases through its effects on different ecological scales (ecozones, biomes and ecotopes) [8]. Climate change would be expected to modify the spatial and temporal distribution of the leishmaniases because the different transmission cycles have long been known to have distinctive “landscape epidemiologies,” as first described by Pavlovskii [9]. The general epidemiological picture is that the leishmaniases are emerging or emergent diseases [10, 11], but these reviews do not specify how climate change will affect the distribution of most of the transmission cycles and diseases. Leishmaniasis is not unusual in this respect: Kovats et al. concluded that there was little clear evidence to associate global or regional climate change with modifications of the epidemiology of arthropod-borne diseases [12]. Notable exceptions came from studies of the impact of yearly seasonal variation in climate. Most transmission of *Leishmania* species is by the bite of the female of permissive sand fly species (i.e., those permitting the development of infective parasites), and so climate change will affect the distribution of leishmaniasis in three ways: (1) directly, by the effect of temperature on parasite development and vector competence; (2) indirectly, by the effect of temperature and other environmental variables on the range and abundance of the sand fly species that act as vectors; and (3) indirectly, through socioeconomic changes that affect the amount of human contact with the transmission cycles.

Concerning the direct effect of climate change, it is known that female sand flies seek sheltered resting sites for blood meal digestion [13], and that temperature affects the development of infective forms of *Leishmania* in sand fly guts [14]. In southern France, microhabitats buffer the variation in air temperature (P.D. Ready, unpublished data), but the temperature changes should be sufficient to influence the development of infective forms of *L. infantum* within the guts of local vectors. Vectorial capacity has only been calculated indirectly. The average number of gonotrophic cycles (i.e., egg development following a blood meal) completed by the vector *Phlebotomus ariasi* in the south of France was only a little greater than one [15], and so relatively small changes in climate could have a large effect on transmission.

12.4 Leishmaniasis and Other Vector-Borne Diseases

Temperature data trend analysis confirms that the temperature regime in South-East Asia (SEA), particularly Bangladesh, is rising following the global trend. Increase in temperature may provide better environments for vector/sand fly/mosquito breeding in places where the temperature was previously below optimum levels. Due to the rise of the temperature to this optimum level, sand fly breeding will increase, and so will the incidence of kala-azar in the human population. Rising temperatures due to climate change will have an impact on the transmission dynamics of kala-azar. Therefore, long-term studies need to be carried out to correlate the rise of temperature, the increase of vector breeding, and the increase of kala-azar. Leishmaniasis is prevalent in 45 districts in Bangladesh as a sand-fly-borne parasitic disease. A population-based survey among 2,348 people showed an incidence of 2% per year from 2000 to 2002, with a case-fatality rate of 19% among adult women, compared with 6–8% among other demographic groups. The current burden of VL is still 20 times higher (21 cases per 10,000 population) than the WHO target level. It has been increasing similarly in other South-East Asia Region (SEAR) countries.

12.5 Temperature Rise, Flooding, Sanitation, and Health Impacts

The overall temperature regime in Bangladesh is showing a rising trend according to the daily maximum and minimum series considered on both an annual and seasonal basis. By one estimate, the average increase in temperature in Bangladesh by the years 2030 and 2075 will be 1.3°C and 2.6°C, respectively, compared with the base year 1990. The seasonal variation in temperature will be greater in winter than in summer, with a 1.3°C increase in winter and 0.7°C in summer for 2030, and 2.1°C for winter and 1.7°C for summer for 2075. Global warming will increase the intensity of the south-west monsoon, which will, in turn, bring about catastrophic ravages like floods and have far-reaching consequences on health. During floods, there is a

problem of sanitation. It has always been found that during and after floods, water-borne diseases increase as a result of heavy contamination of the surface water. If the frequency of flooding increases, this will have an impact on water-borne diseases. Therefore, the ultimate effect of climate change will be more flooding, the break-down of sanitation systems, and more water- and food-borne diseases like soil-transmitted helminthiasis (STH), cholera, diarrheal diseases, dysentery, and so forth. Therefore, the changing climate will ultimately have an effect on human health in Bangladesh, and long-term studies need to be conducted.

12.6 Sea-Level Rise and Its Impact on Health

Various scenarios have been predicted about sea-level rise (SLR) in Bangladesh. Two estimates of potential future SLR for Bangladesh are 0.30–1.5 m and 0.30–0.50 m for 2050 based on the estimation by Bangladesh Department of Environment and Forest in 1993. A very recent study indicates that mean tidal levels at Hiron Point (21°48' N, 89°28' E), Char Changa (22°08' N, 91°06' E), and Cox's Bazar (21°26' N, 91°59' E) are showing increases of 4.0 mm per year, 6.0 mm per year, and 7.8 mm per year, respectively, which is much higher than the global rate. The SLR will impact the health of people living in the coastal area of Bangladesh. Areas measuring about 2,500 km^2, 8,000 km^2, and 14,000 km^2 (corresponding to 2%, 5%, and 10% of the total land area of the country) will be lost as a result of SLRs of 0.1 m, 0.3 m, and 1.0 m, respectively. SLR will cause increased inundation of coastal land areas. This factor, coupled with reduced water flow from elevated areas in winter, will accelerate the intrusion of salt water inland. The salinity of coastal waters and the soil will increase. Even the groundwater aquifers will bear the brunt of salinity intrusion. The increase in salinity in underground water will affect the availability of safe fresh water. As a result, people will be more inclined to use unsafe, contaminated surface water, and will contract various water-borne infectious diseases.

Global water resources both in developed and developing countries have been identified as vulnerable to climate change. One-third of the world's population currently lives in water-stressed countries. It is known that dry lands will become hotter, drier, and more water-stressed as a result of human-induced climate change, and that desertification will potentially affect one-sixth of the world's population. Water quality is also a concern even in more humid areas. Climate change threatens to exacerbate supply problems by increasing the demand for safe water.

If SLR is 45 cm, then the projected potential land loss in Bangladesh will be 15,668 km^2 (11% of total land area), affecting 5.5 million people (5% of total population). Floods are accepted as a major hazard that is likely to increase in some areas because of climate change [1]. Though estimating any effect of global warming is itself a "hazardous" exercise, it is also now increasingly accepted that climate change is likely to cause an increase in flood hazard in many areas of the world. Flooding damages sanitation systems. As a result, the transmission of water- and vector-borne infectious disease including STH will increase. By the year 2030, an additional 14.3% of the country will become extremely vulnerable to flooding, while

the already flood-vulnerable areas will face higher levels of flooding. It is estimated that a 2°C warming combined with a 10% increase in precipitation would increase run-off in the Ganges, Brahmaputra, and Meghna rivers by 19%, 13%, and 11%, respectively. Therefore, SLR will have an impact on human health.

12.7 Climate Change and Livelihood

Water supply, food production, human health, availability of land, and ecosystems are the essential components of the lives and livelihoods of people around the world. Global warming may induce sudden shifts in regional weather patterns such as monsoons. Such changes would have severe consequences for water availability and flooding in tropical regions and threaten the livelihoods of billions. Key links and interactions influence the potential effect of climate change on diseases. Climate change is seen as manifesting itself in each of three interrelated modules: changes in transmission biology, ecologic changes, and sociologic changes that will result in epidemiologic outcomes, including mortality and morbidity rates. Any effects of climate change will probably operate on these groups of factors in different ways, and will likely be nonlinear, region-specific, and time-dependent. For example, climate change may alter the range and abundance of species present in an ecologic community. Nutrient cycle changes, community relocation and biodiversity loss may each affect vector physiology and behavior, vector populations, and vector migration. Another example is that the demographic and economic effects of disease emergence could impact local ecosystems through pollution and habitat loss, which in turn could alter nutrient cycles and deplete species diversity. These effects may also arise from, or be exacerbated by, human migration. As a result, the ability of a local environment to supply nutritional needs may change over time, thus altering people's ability to combat diseases.

The impact of economic development on livelihood indicators, such as nutrition and sanitation, are critical when considering the potential effects of human-induced climate change on health. Increased polarization of societies from economic growth could favor the transmission of diseases because the poor would become more marginalized.

Bangladesh is certainly vulnerable to the consequences of climate change. SLR and the associated salinity of agricultural lands in coastal areas, along with shortages of fresh drinking water, are likely to be the immediate outcomes of global temperature increase. The drying-up of glacial melt waters from the Himalayas will greatly reduce the water flow in the Ganges, which is one of the main resources for fresh water. Bangladesh is also prone to water-related events like flooding, heavy rainfall, or droughts that significantly impact the health and livelihood of the population. Not only subsistence agriculture will be affected adversely; the food security of the poor will be at risk. They will face adverse health effects due to outbreaks of pathogen-driven and water-borne diseases. Their settlements will deteriorate, and their overall quality of life will be diminished. The effect of climate change on peoples' livelihoods will be disproportionately felt by the poor of Bangladesh. In addition, given

its location at the confluence of the deltas of three great rivers, its history has always been one of extreme environmental vulnerability. Poverty, low literacy rates, poor infrastructure, and inadequate government capacity ensure high levels of vulnerability, as well. Frequent disasters might trigger migrations from affected rural areas, and will create ecological refugees within Bangladesh. Given all the ways climate change can impact livelihood, it is crucial to develop adaptation strategies to cope with the changing scenarios that might face the vulnerable communities of SEAR.

12.8 What Should SEAR Countries Do About Climate Change?

In December 2007, SEAR countries, with the support of WHO, prepared a regional action plan to protect human health from the effects of climate change. The goal of the regional action plan is to build capacity and strengthen health systems [4]. The first step will be to increase awareness of health consequences of climate change by:

1. Undertaking studies on the health implications of climate change and sharing information to understand how to promote changes in individual and corporate behaviors that mitigate climate-related health risks, while protecting and promoting health.
2. Facilitating national working groups, non-governmental organizations (NGOs) and civil society to develop coordinated mitigation and adaptation plans, including relevant sectors, regions, and disciplines.
3. Developing awareness-raising programs and learning materials to educate and engage a broad range of stakeholders, including local communities, health and other professionals, and the media on the potential health impacts of climate change and on appropriate measures to reduce climate-sensitive risk factors and adverse health outcomes.

To strengthen health systems capacity to provide protection from climate-related risks, and to substantially reduce health systems GHG emissions, SEAR countries will:

1. Develop and implement national action plans for health that are integrated with existing national plans for adaptation to and mitigation of climate change.
2. Develop integrated strategies to incorporate current and projected climate change risks into existing health policies, plans, and programs to control climate-sensitive health outcomes, including integrated vector management and disaster risk management.
3. Strengthen existing infrastructure and interventions, including human resource capacity, particularly surveillance, monitoring, and response systems and risk communication, to reduce the burden of climate-sensitive health outcomes. Key concerns vary by country; common concerns include vector-borne diseases, air quality, and food and water security.

4. Strengthen public health systems and disaster/emergency preparedness and response activities, including psychosocial support, through increased collaboration and cooperation across sectors. This should include documentation, sharing, and evaluation of the effectiveness of local knowledge and practices.
5. Provide early warning systems to support prompt and effective responses to current and projected health burdens. To achieve this end, national and regional climate forecasting information, including climate change projections, should be fully utilized.
6. Implement adaptations over the short-, medium-, or long-term; be specific to local health determinants and outcomes of concern; and facilitate the development of community-based resource management. The costs and benefits of different interventions should be determined.
7. Establish climate change focal points or mechanisms within the national health institutions to ensure the implementation, monitoring, and evaluation of health mitigation and adaptation actions, and to ensure that health issues are adequately addressed in these actions.
8. Establish programs to substantially reduce GHG emissions by the health sector which could also serve as a best practice model for other sectors.

To ensure that health concerns are addressed in decisions to reduce risks from climate change in other key sectors, the following activities will be carried out:

1. Develop integrated strategies to incorporate current and projected climate change risks into existing policies, legislation, strategies and measures of key development sectors to control climate-sensitive health outcomes.
2. Facilitate the health sector to actively participate in national communications to the United Framework Convention on Climate Change, and include health issues as the core elements in the negotiation process.
3. Ensure active health participation in the national climate change team.

12.9 Conclusion

Finally, we would like to stress that the impact of climate change on kala-azar is unclear. This is because of the absence of studies of sand fly ecology in relation to climate change in Bangladesh. To start with, information should be gathered about current sand-fly distribution. Flooding might kill off the sand fly population and thus be beneficial from the point of view of disease control in the case of kala-azar. Catastrophic flooding or SLR may cause people to migrate to non-flood areas, further increasing population density. This would significantly increase the risk of infectious diseases including kala-azar. Emphasis must be placed on reducing the overall vulnerability of people to the impact of climate change. Bangladesh offers an excellent disease surveillance system for studying the effects of climate change. Without reliable base data, it is impossible to determine and predict the impact of climate change on kala-azar.

Appendix: What Can We as Individuals Do to Help Reduce the Adverse Health Impact of Climate Change?[1]

Act now for climate change.

Buy the most energy-efficient model appliances such as washing machines, refrigerators, dishwashers or ovens, office equipment such as computers, photocopiers, and printers.

Calculate our personal carbon footprint and cut our GHG emissions.

Discuss and distribute informational materials on climate change and environmental health issues.

Enjoy the sun, fit solar panels on the roofs of our homes, turn our homes and/or offices into clean power stations with solar power that is renewable.

Fridge doors should not be kept open for longer than necessary; let food cool down fully before placing it in the freezer; defrost regularly and keep the appliance at the right temperature. Where possible, don't place ovens/cookers and fridges next to each other.

Go buy a fuel-efficient, environmentally friendly car, if possible. It will save money and keep more CO2 from going into the atmosphere. Make sure that the tires are inflated correctly; this can save 5% on the cost of fuel. Share car rides (carpool) with our work colleagues or friends. Make more use of public transportation for longer trips. For short trips, try walking or use a bicycle; this keeps us physically fit.

Halve emissions by moving our air conditioner thermostats up by 2°C in summer. Almost half the energy we use in our homes goes to cooling. Maintain the filters on air conditioners, cleaning them regularly. A clean air filter can save pounds of CO2 a year.

Involve our family, friends, children, and neighbors.

Join or start an environmental group to start action in our locality or region

Kick – start an environmental campaign in our neighborhood.

Light bulbs: replace light bulbs with fluorescent bulbs. They cost more than ordinary light bulbs and also use only about one-quarter of the electricity to provide the same light. And they last four times longer than normal light bulbs.

Minimize the use of toxic chemicals. Use non-toxic, bio-degradable, water or plant-based paints, cleaners, and pest repellents.

Network with specialized agencies, NGOs, and engaged communities.

Off: Turn off televisions, videos, stereos, and computers when they are not in use – they can consume between 10 and 60% of the power when in "stand-by" mode. Turn off computer screens and photocopiers during breaks. Also turn off lights when not needed; it saves energy after a minute or two. Unplug electronic items when not using them.

Plant trees and launch tree-planting campaigns in our communities.

[1] Adapted from reference 4.

Quit using plastic bags and bring our own bags when shopping.

Recycle, repair, reuse materials. Collect and share tips, tricks, and ideas for sustainable living.

Save paper, print on both sides of the page. Proofread documents on-screen before printing. Instead of making a copy for each person, route one copy around the office. Do not discard one-sided printed pages; use them to make scratch pads.

Traveling by plane should be reduced as it contributes to significant CO2 emissions leading to climate change. Consider buying carbon offsets to compensate for the emissions caused by flights.

Use less energy and conserve more of it. Do not waste water while brushing or washing clothes, body, or dishes. Repair leaky plumbing fixtures; prevent overflowing of tanks. Energy is used for pumping and treating water. Save water to save energy.

Value wastes, do not dump home wastes everywhere. Heaps of garbage left in the open emit methane and contribute to global warming. Segregate wastes so they can be recycled and/or reused and, where possible, use organic waste for composting.

Write about the health impact of climate change in newspapers, a great way to keep the issue in the public eye. It also sparks debate and allows us all to understand the real issues.

X*press your concern on environmental health issues and solutions and stay informed.* Read widely and understand what we are dealing with.

Y*our government leaders – president, prime minister, parliamentarian, senators, and/or mayors, etc.* – all need to know about the different ways climate change has impact on health. Write letters to them asking for policies to ensure GHG emissions fall by at least 3% each year from now on.

Z*oom in on reducing emissions. It is the best way forward.* Countries need new national legislation and laws to help ensure that we develop cleaner cars and cleaner power plants, and to help us get government rebates on installing solar power, solar hot water, or wind power in our homes.

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Chapter 13
The Role of Policy Makers in Achieving the Target for Kala-Azar Elimination in South Asia: The Bangladesh Experience

Kazi M. Jamil

Abstract Kala-azar is one of the world's most neglected diseases, affecting millions of poor people in South Asia. In spite of an abundance of medical literature and surveillance reports citing the incidence and severity of the disease, mortality and morbidity due to kala-azar have been continuing unabated for many decades in this region. A dramatic turn of events was noted after the health ministers of India, Bangladesh, and Nepal signed a historical memorandum of understanding in 2005 to eliminate the disease, thanks to the diligent efforts of the World Health Organization's South-East Asia Regional Office. The "neglect" was apparently overcome, but much remained to be accomplished: a target was set to reduce the incidence of kala-azar to less than one per 10,000 population at subdistrict levels in the endemic areas within a decade. The regional strategy was based on mandatory use of the rK39 rapid diagnostic test for diagnosis combined with the use of the oral drug miltefosine. A phase 4 trial on miltefosine was conducted by the Ministry of Health in Bangladesh, and miltefosine was introduced for the treatment of kala-azar in 2007, but the drug disappeared from the market the following month. The drug controller had to withdraw miltefosine because it turned out to be ineffective and to lack active ingredients. Another dimension of "neglect" was thus unveiled that jeopardized the lives of millions seeking medical care. The responsibility to identify and eradicate the flaws in the drug approval and distribution system lies with the policy makers who kindled hopes of eliminating this dreadful disease.

Keywords Bangladesh · Kala-azar elimination · Kala-azar policy

Abbreviations

IRS	Indoor residual spraying
MOU	Memorandum of understanding
RTAG	Regional technical advisory group
SSG	Sodium stibogluconate

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6_13,

TWG Technical working group
WHO World health organization

13.1 Introduction

Neglected tropical diseases are a great challenge for developing countries, as they affect an estimated 2.7 billion people living on less than US$2 per day [1]. In spite of the availability of cost-effective treatment regimens, national and international efforts to control these diseases have been minimal until recently. The burden of kala-azar is described in detail in Chapter 1 by Moazzem Hossain and Kazi M. Jamil, in this book, which underscores the importance of eliminating the disease from South Asia. The present chapter highlights the evolution of the kala-azar elimination program recently launched in South Asia and will attempt to identify its strengths and weaknesses as well as offer a critical assessment of the role of policy makers in overcoming obstacles to achieve the program's goals.

13.2 The "Regional Strategy" for Eliminating Kala-Azar

India, Bangladesh, and Nepal expressed a commitment to eliminate kala-azar from the Indian subcontinent by the year 2015 during a meeting of the health ministers of the South-East Asia Region countries held on the Maldives on September 5–6, 2004. A Regional Technical Advisory Group (RTAG) for kala-azar elimination was formed to develop a regional strategic plan to achieve kala-azar elimination in the three countries. The RTAG made the following recommendations: (1) Kala-azar should be eliminated from these three endemic countries by the year 2015. (2) The World Health Organization (WHO) should assist by increasing access to the population at risk. (3) Country-specific plans for eliminating the disease should be developed within the framework of the regional strategic plan by using the regional guidelines for preparing national plans. WHO should provide technical support in the preparation of national plans for the elimination of kala-azar. (4) The elimination program should be directed to emphasize

- Early diagnosis and complete treatment that is operationally feasible, to ensure outreach to the poorest (e.g., appropriate dipstick diagnosis and safe and effective oral medicine);
- Strengthening disease and vector surveillance programs through an efficient, built-in management information system that will provide a network for all reporting facilities;
- Vector control through integrated vector management with a focus on indoor residual spraying (IRS), insecticide-treated nets, and environmental management;
- Social mobilization directed toward behavior change through effective communication strategies;
- Clinical and operational research to support the elimination program.

Subsequently a memorandum of understanding (MOU) was signed by the ministers of health of the three countries on May 18, 2005, during the 58th World Health Assembly held in Geneva, containing a pledge to eliminate kala-azar from their respective countries. This was followed by a number of expert meetings under the leadership of WHO's South-East Asia Regional Office. Two valuable documents resulted from a series of meetings organized by WHO: (1) Regional Strategic Framework for Elimination of Kala-Azar from the South-East Asia Region (2005–2015), and (2) Regional Framework for an Integrated Vector Management Strategy for the South-East Asia Region [2, 3]. Four major components of the "Regional Strategy" were described in these documents: (1) early diagnosis and complete case management; (2) effective disease and vector surveillance; (3) integrated vector management with a focus on IRS; and (4) social mobilization and building partnerships.

13.3 Implementation of the "Strategic Plan" in Bangladesh

The Technical Working Group (TWG) for the kala-azar elimination program was assigned to revise the national strategic plan in 2008 in the context of local needs. A large group of experts (medical doctors, microbiologists, entomologists, educationists) were invited to work together with members of the TWG to develop a "training module" and updated "national guidelines" for the diagnosis and treatment of kala-azar in Bangladesh [4]. In spite of strong pressure from WHO to stop the use of sodium stibogluconate (SSG), it was decided that SSG would not be discontinued until alternative drugs became available in adequate supply. The foresight shown by these experts was revealed in later years when SSG remained the only drug available for the treatment of kala-azar after miltefosine was discovered to be ineffective despite a successful clinical trial – a development described in later sections of this chapter. The new training module was immediately used to develop trained manpower – including doctors, nurses, health workers, entomologists, and exterminators – and to prepare them to implement the national strategic plan for eliminating kala-azar. The program reported remarkable progress by the first quarter of 2008 [9], as enumerated below:

1. Training of the master trainers (doctors) for the kala-azar elimination program;
2. Training of nurses, medical technologists, and health workers to be deployed in endemic areas;
3. Training of master trainers (exterminators) for insecticide spraying, and training of workers to be deployed in the field;
4. Advocacy meetings with opinion leaders, teachers, and students, as well as religious leaders (*imams*);
5. Procurement of drugs, insecticides, rapid diagnostic test (RDT) strips, spraying equipment, etc.;
6. Development and printing of information, education, and communication materials;
7. Development of materials for TV and radio broadcast, and implementation of the same.

The hard work of the trained and motivated teams deployed in endemic areas soon paid off. The most important of these achievements were: replacing the aldehyde test with RDT in every endemic upazila or subdistrict; following national guidelines for treating all kala-azar cases in upazila health complexes; and starting IRS as a pilot trial in some endemic areas.

13.4 The Rise and Fall of Miltefosine in Bangladesh

Making miltefosine available as an oral drug for kala-azar has been an avidly-pursued goal in the tropical disease research program at WHO, which promoted the development of this drug for many years. Following a phase 4 trial of miltefosine in Bangladesh that was never published, the drug was introduced by the program in the first quarter of 2008. It became apparent within 1 month that the drug was ineffective, and death was reported in some patients who were treated with the new drug. Miltefosine had to be withdrawn from the market [5]. No words can describe the disappointment among the highly-motivated team that had launched the war against kala-azar in Bangladesh under the able leadership of the Disease Control Unit of the Directorate General of Health Services at the time when this unfortunate event occurred. How could such a thing happen? Who was to blame? How can incidents of this sort be prevented in the future? It took many months to receive an official report from WHO that confirmed that the drug used in the program had in fact contained no active ingredient. It is worth noting that Bangladesh still lacks the capacity to test a drug like miltefosine for quality assurance.

13.5 The Need for a Quality Assurance Program for Drugs

The rapid progress shown by the kala-azar elimination program in Bangladesh in the first few years after signing the MOU with neighboring countries demonstrated the capabilities of the country's disease control unit. The unexpected "miltefosine case" was a lesson learned and should not be forgotten. It has clearly shown the weakness in the system that must be mended without delay. At least two changes should be implemented to prevent future occurrences of this sort. First, a drug testing facility should be established to monitor the quality of drugs being used in the program. This may be achieved through an effective partnership among the stakeholders who have spearheaded the elimination program in South Asia. Second, strict laws must be enacted and enforced, and prompt disciplinary action taken against the parties responsible for compromising drug quality.

13.6 Partnerships for Utilizing All Available Resources

Yet other policy issues hindered the implementation of WHO recommendations for the kala-azar elimination program in Bangladesh. The government's procurement rules did not allow the program to purchase rK39 dipstick tests (KalazarDetect;

InBios, Seattle, WA, USA) and deltamethrin (a pyrethroid), although both the rK39 test for the rapid diagnosis of kala-azar and pyrethroids for vector control are widely regarded as cornerstones of kala-azar elimination. Fortunately, the International Centre for Diarrhoeal Disease Research, Bangladesh came forward to evaluate RDT kits (CTK Biotech, San Diego, CA, USA) procured by the government, and found both their sensitivity and specificity to lie above 90% (Jamil et al., unpublished). This is probably the only instance of the Ministry of Health entering into an effective partnership with a non-government research institution to achieve the goals of the kala-azar elimination program.

13.7 Improving Surveillance of Kala-Azar and Post-kala-azar Dermal Leishmaniasis

The present surveillance system needs to be strengthened in at least two respects. The private sector plays a significant role in providing health care almost everywhere in Bangladesh but remains completely outside the loop of the surveillance program. Private practitioners should be encouraged to participate by reporting suspected cases of kala-azar and providing treatment when feasible. The need for developing an effective system for reporting post-kala-azar dermal leishmaniasis (PKDL) is discussed in Chapter 11 by Philippe Desjeux and V. Ramesh of this book.

13.8 Strengthening Vector Control Programs

Very little progress has been made with IRS in Bangladesh despite the clear emphasis on vector control activities outlined in the national strategic plan. However, the policy issues mentioned above regarding the procurement of the recommended insecticide must first be resolved. Insecticide-impregnated bed nets should be promoted in endemic areas to achieve vector control. The procurement of supplies should be coordinated with the training of health personnel to reach vector control goals. A surveillance system should be put in place for monitoring the success of the vector control activities so that any emergence of resistance to the insecticides may be detected promptly and appropriate action taken.

13.9 Future Directions for Basic and Operational Research on Kala-Azar

The development of a human vaccine to protect against kala-azar may seem to be a long way off, but a therapeutic vaccine has been proposed for the management of PKDL [6]. Combination therapy for kala-azar urgently needs to be evaluated to reduce the duration of treatment and curb the development of resistance to the small number of anti-leishmanial drugs on the market. Improving mass awareness of kala-azar in endemic areas is essential for reaching the goal of kala-azar elimination.

Knowledge of kala-azar was found to be alarmingly low in endemic areas, and was lowest of all in Bangladesh as compared with India or Nepal [7]. All the wonderful new medical advances for the elimination of kala-azar will be in vain if the victims remain unaware of the disease and fail to seek help for its treatment and prevention. Operational research should be carried out to find the most effective approach to enhancing awareness in the community. School-based health programs have been found to be effective in other parts of Asia [8, 9] and deserve serious consideration. The government should encourage public-private partnerships as they have proven successful in other control programs including those for tuberculosis, malaria, avian flu, and so forth. The government may provide funding for research either directly or indirectly by providing tax exemptions to commercial organizations that will invest in research relating to the national strategic plan for the elimination of kala-azar.

13.10 Conclusion

The Bangladesh experience with its kala-azar elimination program demonstrates that much of the success of the multi-country efforts to eliminate this disease from South Asia will depend on policy makers in these nations. Amendments should be made to existing procurement policies to ensure the availability of high-quality drugs and supplies for the program in Bangladesh. The necessary infrastructure should be developed without delay to assure the quality of drugs and other supplies for the control program. Opportunities for public-private partnerships should be explored to make the best use of available resources. Finally, an integrated approach should be adopted as the most cost-effective way to eliminate kala-azar and other parasitic diseases in South Asia.

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T.K. Jha, E. Noiri (eds.), *Kala Azar in South Asia*,
DOI 10.1007/978-94-007-0277-6, © Springer Science+Business Media B.V. 2011