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Special Issue on Post Kala-azar Dermal Leishmaniasis

Guest Editor:

Mitali Chatterjee,

MD PhD FNA FASc FNASc FAMS FRCP FTWAS

Professor of Pharmacology, Institute of Post Graduate Medical Education & Research

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Addresses

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Dr. Atanu Biswas,
Editor in Chief,
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Volume No. 4 Special Issue No. 1 June 2025

CONTENTS

Original Articles

Editorial

The imageries of ancient Greek mythology has permeated the broader culture of scientific enterprise. All the planets of our solar system are named after Greek and Roman gods or goddesses, except for the Earth. Charon is an important character in Greek mythology who bridged the gap between life and death, ferrying the souls of the dead to the underworld. According to Roman myth, when one traveled to the underworld, one needed to cross the river Styx in Charon's boat paying a toll. Pluto, the ruler of Greek underworld, presided over the afterlife and Charon was his close associate. Five mythical rivers were believed to encircle Pluto's underworld, and the name Charon is often assumed to be euphemism for death. From the nineteenth century, deaths due to an unknown disease that raged ferociously killed millions in the Indian sub-continent, later named as Kala-azar or black fever.

A young physician and scientist Dr. Upendranath Brahmachari (1873–1946) in 1922 appeared like a messiah to ward off Charon's toll by discovering an effective chemotherapeutic against kala-azar, Urea Stibamine, long before the discovery of penicillin. It may be recalled that urea stibamine was the second chemotherapeutic in the world against infectious diseases after Paul Ehrlich's anti-syphilitic drug, Salvarsan (compound 606) discovered in 1909. Brahmachari's reearch was a unique blend of chemistry and medicine and his work stands as a monument to his labor, dedication, and expertise and amply reflected in the clinical success it attained.

The drug effectively countered the epidemic of kala-azar during the late twentieth century in the vast tracts of the Gangetic plain and the Brahmaputra valley. The wonder drug turned the tide from a 90 % death rate to a 90 % recovery, yet remains unheralded, like so many other Indian scientists who achieved stupendous success in their fields during colonial rule. Dr. Brahmachari also identified a variant of kala-azar characterized by skin eruptions appearing after clinical recovery. In 1922, he named this condition post-kala-azar dermal leishmaniasis (PKDL).

Even a century after Sir U.N. Brahmachari's discovery of PKDL, the condition remains an unresolved enigma, particularly why 10% to 20% of kala-azar cases in South Asia develop PKDL. The importance of PKDL cannot be underestimated, as in all likelihood, it is the strongest contender to be the disease reservoir of kala-azar, and remains a major stumbling block for the elimination of kala-azar. Furthermore, Leishmaniasis continues to widen its base as small foci sporadically appear in different parts of the country, as reports of a new variant of *Leishmania donovani* have surfaced in Himachal Pradesh. The new variant shows uncommon dermal manifestations, rather than the usual viscera tropism.

This special issue focuses on some recent scientific advances in *Leishmania* research and also offers glimpses into the early history of kala-azar in the Indian subcontinent. It covers a wide range of topics, from the discovery of the causative agent *L. donovani*, the chronic nature of the disease, and its transmission, to the bumpy road towards identifying the vector *Phlabotomous argentipes*, the treatment modalities, resistance mechanisms, and immunopathogenesis. It aims to find a reasonable conclusion to whether PKDL is a new disease, or whether it develops due to refractoriness of failure to existing treatment regimens.

Infectious diseases have impacted human civilization, but the history of kala-azar holds particular significance for us, as much of its pioneering research was conducted in this city, Calcutta, contributing profoundly to the scientific landscape. Medical history not only teaches us the origins of such diseases but also helps us understand our current position and the path ahead in combating them. It reminds us of T S Eliot's famous lines,

> 'Time present and time past
> Are both perhaps present in time future
> And time future contained in time past'

We hope this special issue that offers both a historical portrait of kala-azar and the current state-of-the-art understanding of kala-azar and PKDL will serve the needs of the present scientific community, as well as inspire those who are yet to enter this field of research. We are extremely delighted to acknowledge the contribution of our colleagues from different corners of the world to make this special issue a "must read" for fellow Leishmaniacs and beyond.

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Anidrita Saikia, Mitali Chatterjee[1], Syamal Roy[2]

Department of History, Delhi University, New Delhi, Delhi, [1]Department of Pharmacology, IPGME&R, Kolkata, West Bengal, [2]Emeritus Scientist, Indian Institute of Chemical Biology, Kolkata, West Bengal, India

Address for correspondence: Prof. Mitali Chatterjee, Department of Pharmacology, IPGME&R, Kolkata 700020, West Bengal, India.
E-mail: ilatimc@gmail.com

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Review Article

Kala-azar and the contestations of its nomenclature

Anidrita Saikia

Department of History, Delhi University, New Delhi, Delhi, India

Abstract

This review examines how the epidemic of kala-azar was recorded in the medical registers of Assam under various debated names, stirring the British colonial state to trace its origins and its identity. Inquiries were focused on establishing the origin and cause of this mysterious fever as also the associated "darkening of skin," the latter possibly being emphasized with a view to shifting responsibility from the colonial state to the local environment and individuals residing in those regions. The government led research tended to attribute the fever to the "insalubrious" conditions, whereas the locals termed it as "sarkari bemari" or "governments disease" placing the onus on their masters.

Keywords: Colonial health policy, kala-azar, sarkari bemari

Address for correspondence: Ms. Anidrita Saikia, Department of History, Delhi University, New Delhi 110021, Delhi, India.
E-mail: rukmisaikia0307@gmail.com
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INTRODUCTION

In the summer of 1896, a young man named Badal arrived at the civil dispensary in Nowgong, a station town in the Brahmaputra Valley of Assam, with the complaint that his skin was turning black.[1] The 25-year-old, who had only been in Assam for four months and complained of fever and fatigue. He was admitted, staying in the dispensary for three months under medical supervision and steadily improving, discharged in September in much better shape.[1] The darkening of the skin that troubled Badal was not simply a change in the skin color. In English, darkness means the state of absence of light, but in vernacular Assamese, as well as Hindi, the word dark, pronounced as "kala" or "kola," is equated with blackness. However, in the medical registers of nineteenth century Assam, it carried a far more sinister and perilous connotation. The changing of the skin to dark – or "kala" – was a symptom of a disease that was called "kala-azar" which translates to black fever. Today, kala-azar, leishmaniasis, is one of the few diseases whose South Asian name is still included in medical text books.

From the late nineteenth century, the region of the Garo Hills, the Brahmaputra Valley of Assam, and the Cachar belt came to be embedded within the identity of this disease that shouldered the threats of uncertainty and deviancy. Kala-azar, synonymously called black fever and Assam fever, was a word that evoked anguish and horror among locals and British alike [Figure 1]. The disease became the cornerstone of medical discourse, with physicians arriving in Assam to conduct extensive investigations on its "dubious" causes and "shadowy" means of transmission. It was only in the 1910s that conclusive answers started emerging. The British state grew increasingly anxious, even though the disease hardly affected the Europeans, as absent villages meant loss of a

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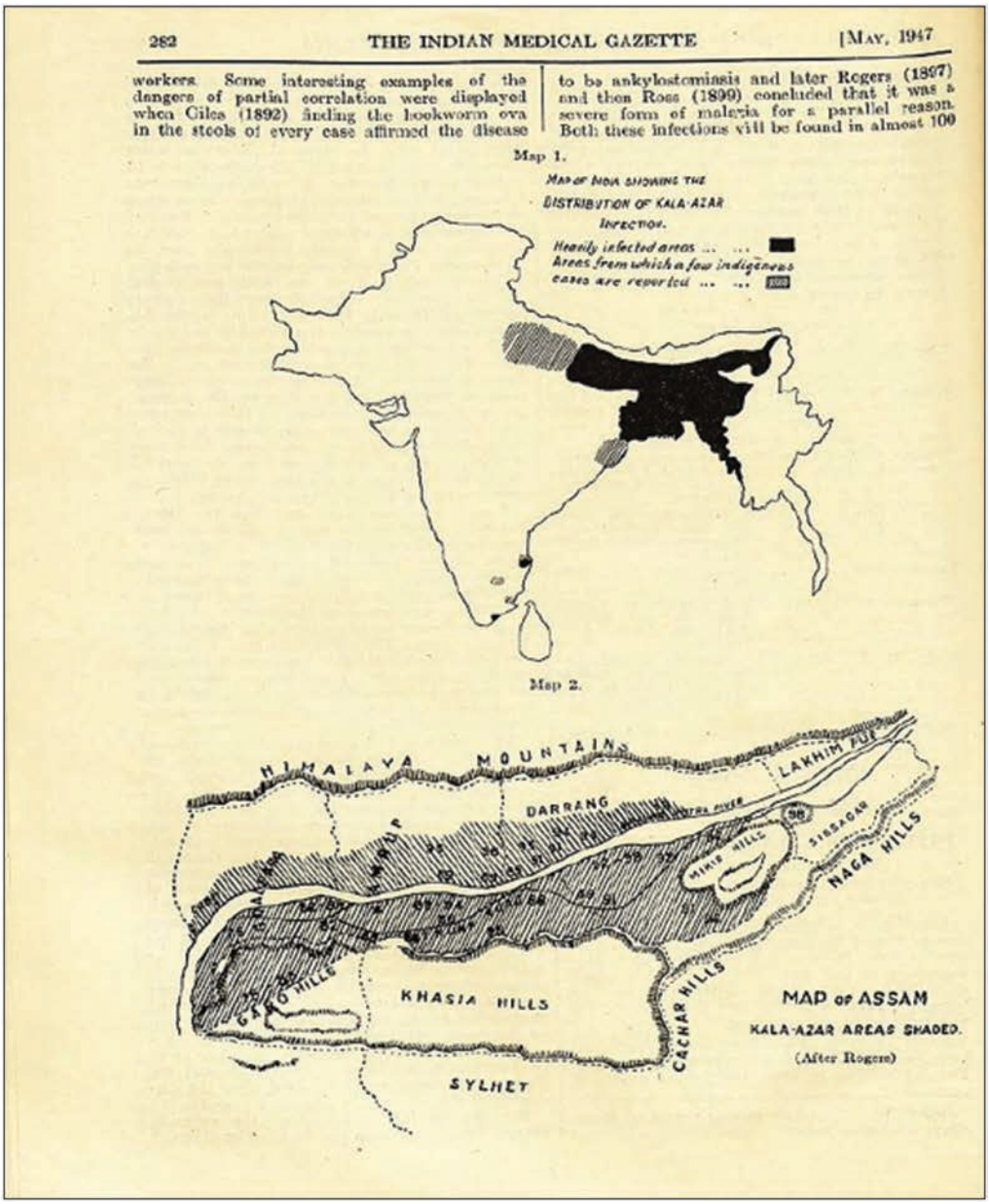

Figure 1: Map showing distribution of kala-azar infection

source of revenue, thus striking at the heart of the state's political economy.

How did the disease acquire an identity bound to pigmentation, when its symptoms encompassed so much more? The medical discourse pointed to an entanglement of assertions bound with administration, economy, and a civilizational narrative that tended to emphasize British superiority. The disease kala-azar was codified in the medical registers of Assam, with different names that prompted debates and research aimed at tracing the identity of the epidemic.[2] How was the disease caused and transmitted? Tracing the genesis of the disease meant reaching an answer as to how, when, and where the disease stemmed from, and finding a conclusive answer proved challenging. The symptoms transgressed the borders of clinical features of malaria, kala-azar, and the search for an identity unraveled the bias of pathogens being labeled as "dense" and "insalubrious". Much like other provinces, Assam had been stigmatized as "unhealthy" even before the epidemic emerged.[2]

The darkening of the skin that Badal complained of was a symptom that evoked a vociferous debate. While the term "kala-azar" is said to have been taken from the Garos, a community inhabiting the Garo Hills of what is today's Garo Hills, its etymological origins were not to be found in the Garo language [Figure 2]. A more common

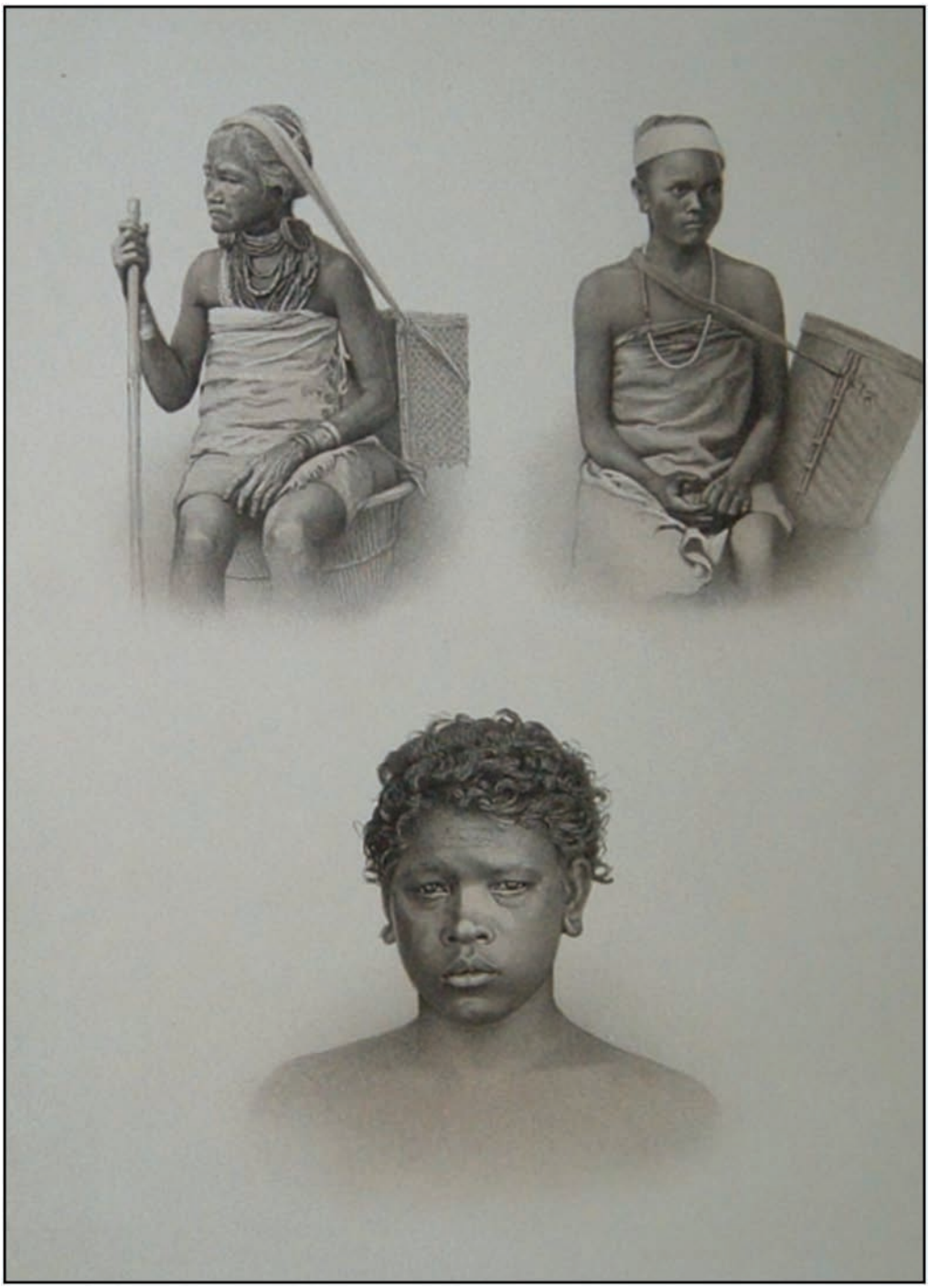

Figure 2: The term "kala-azar" was initially taken from the Garos, a community inhabiting the Garo Hills

name in these hills was "sarkari bemari" or "sahab's bemari," which translated to "government's disease" or "white man's disease." The name "kala-azar" pinned the identity of the disease on the reported "blackening," yet the terminology of "sarkari bemari" placed the blame squarely on the British. It was no wonder that it irked the officials, as evident in a disgruntled British official G.H. Fink who wrote, "In some villages, the term kala-azar is not known. You hear the Garo word 'Rapungi,' meaning jungle air and water – mist in fact, – which shows that the Garos themselves recognize that the malaria of the jungle has some close connection with the disease known as kala-azar. The term kala-azar or 'kala-zar' is of more recent importation into the Garo Hills and is an Assamese word. Then, again, in some places nearer the plains, you hear it called 'sarkari-bimari,' a term which was doubtless used with a certain amount of cunning when we first occupied the Garo Hills."[2]

Bodhisattva Kar writes about the British dissatisfaction at the disease being termed "sarkari bimari" as it pinned the onus squarely on the state and the economy of the tea gardens.[3] Associating disease through the plantation economy altered the image of the tea gardens that were considered as a harbinger of prosperity, progress, and civilization. However, the disease in its inception did not find an appearance in the tea plantations, being confined to the Garo Hills. Faced with an increasing number of deserted villages in the Garo Hills, it became critical to establish the disease's identity which led to the government seeking the advice of medical experts.[3]

In 1888, Surgeon Captain G.M. Giles, the civil surgeon of Hoshangabad, was assigned by the British state the duty of enquiring into the "nature and origin" of kala-azar.[4] He submitted his report two years later and, based on his studies from the kala-azar cases from the dispensaries of Gauhati, Shillong, and Dibrugarh, confidently posited his findings that kala-azar was the same as "beri-beri" declaring that "beri-beri," the "kakke" of Japan,[5] was one of the two diseases rampant among the tea garden coolies. The first, Ancylostomiasis, was caused by the roundworm Ancylostoma *duodenal*, while beri-beri resulted from a deficiency of vitamin B1 (thiamine).

Giles' report insinuated the dissonance between the state and kala-azar, as he asserted that the "sarkari bemari" was not a problem of the state, but more an affliction of the diseased body within the locality, a body prone to disease bound by exploitation, impoverishment, and an extracting work regimen. Another hypothesis for the origin of the "sarkari bemari" was proposed to be from Rungpore in Bengal following a period of famines in the 1860s and 1870s.[1]

After Giles' inability to converge kala azar and anchylostomiasis thoroughly, Leonard Rogers in 1896 was specially requested to examine this febrile disease which by then had become so terrifying that the natives were rumored to burn their sick brethren alive.[6] Rogers wrote that up till 1875, "there had been no loss of revenue due to the ravages of the epidemic malaria, first called kala-azar in the Garo Hills."[1] Debates sprung up between surgeons and officials on where the disease sprung from, whether it was "Bengal Kutta near Dhubri" or "Karaibari Mahal," but it was agreed by most sanitary officials that the origins lay in the plains of Eastern Bengal, which "came across from Rungpore, and not out of the hills themselves."[1] Rogers writes that "kala-azar might possibly be a continuation of the epidemic of malarial fever of the sixties and seventies in Lower Bengal, which is generally called the 'Bardwan fever'."[1] The Burdwan fever, much like the Assam fever, was a nomenclature that merged geography with disease. Rohan Deb Roy points how both the diseases were epidemics attributed to malaria.[7] Despite the similarities of the febrile disease, the possibility of Assam fever being the same disease as Burdwan fever was eradicated after "a minute study of all the material available, together with a tabulation of such mortality returns and meteorological data as were recorded, and a comparison of these with the more accurate figures of recent years, has shown that the fever epidemic in the north-western part of Bengal in the seventies was independent of the 'Bardwan fever,' and was started by a succession of several years of very deficient rainfall, such as is to this day commonly followed by an unusual amount of fever in these parts."[1]

Dr. Rai Gopal Chandra Chatterjee Bahadur in Calcutta reminded everyone how the disease "remained so long unrecognized as a distinct entity, even after the advent of the bacteriological era, and even after a name has been coined by the uncivilized people of Assam, who gave the name kala-azar or kaladukh to it."[8] Tropical medicine expert Sir Ronald Ross, who arrived in Assam in 1899 to study kala-azar, was furnished with information about vocabularies by doctor administrator L.A. Waddel: "Kala (Sanscritic) means black or deadly. Dukh (Sanscritic) means suffering or pain; literally disease, that is, want of ease. Jwar (Sanscritic) means fever, that is, raised bodily temperature. Azar (Assamese) means sickness; but in Nowgong, the words jwar or bimar (Urdu), illness, are more frequently used by the people. The popular use of the adjective kala does not appear to me to imply necessarily blackening of the skin. Perhaps, the original meaning was deadly. It is possible that the meaning has now become transferred to the more literal significance of the adjective black. Thus, "black death" meant only plague originally until the word stimulating the imagination, people saw a blackening effect in the disease which it does not possess. Just, possibly, the same thing has happened in kala-azar."[9]

In a colonial discourse, the primary connotation of "blackness" is unsurprisingly that of race. Writing about a suspected case of kala-azar in a nine-year-old girl who was "dark in complexion," Giles diagnoses symptoms as malaria, dismissing the change in her blackened skin and remarking that "…she has [not] become blacker than usual."[5] How black – or dark – can someone who is already dark be? In another case of a 23-year-old man, who was brought in by his brother, complaining that "he has become black," Giles refuted again, taking note of the patient's "light brown color" complexion, and pointing out how "he is very fair for a native, and much fairer than this own

brother of his."[5] The British surgeon, for whom "kala-azar of course means black fever," lacked a clear definition of what this "blackness" entails within a diseased body.

If Giles refuted the darkening of the skin as a symptom in 1890, Leonard Rogers asserted that the color of skin changed. In his 1898 report, he remarks that " the disease is characterized by intermittent or remittent fever, with marked general wasting of the body, but with great enlargement of the spleen and liver, edema of the feet, …anemia, great weakness, and often a darkening of the skin, from which the disease probably gets its name of black fever.[10] He asserted that "the comparatively dark skin is often a noteworthy feature,"[10] and while the disease makes the patient anemic, where partly "the natural pigment of the skin being more apparent," there is still "a deposit of fresh pigment in the integument, which is derived from the hemoglobin of the broken-down red corpuscles which made the skin dark."[10]

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published, and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Review Article

Tracing the vector

Anidrita Saikia

Department of History, Delhi University, New Delhi, Delhi, India

Abstract

The initial theory of miasma and malnutrition being responsible for kala azar was dismantled by researchers who identified the causative parasite as *Ancylostoma duodenale*, suggesting the disease was naturally present in the human body. Further research even considered soil as a potential source of the febrile nature of kala azar, but eventually, investigations in the early twentieth century focused on insect vectors as the likely cause of transmission. These enquiries were advanced under the aegis of the Kala Azar Commission, which initially explored the possibilities of the bed bug and the biting midge as possible vectors. Finally, in 1942, after a search that spanned almost 50 years, it was conclusively established that the *Phlebotomus argentipes* sandfly was the vector.

Keywords: Early twentieth century research, kala azar, sandfly, vector identification

Address for correspondence: Ms. Anidrita Saikia, Department of History, Delhi University, New Delhi, Delhi 110021, India.
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MOSQUITO MADNESS

When kala azar was first documented in Assam, it commonly presented as a low grade fever attributed to miasma arising from a humid, tropical climate, with dense jungles and putrid air in the region. This closely aligned it to malaria under the umbrella of the miasma and contagion are two different concepts. It should be miasma theory alone.[1] The first kala azar report disrupted this debate on miasma that the disease was borne out of "putrefaction." In 1888, Civil surgeon G.M. Giles was assigned to investigate the disease in Assam, and he concluded that kala azar was ancylostomiasis (hookworm) caused by *Ancylostoma duodenale*.[2] Parasitism was viewed as "natural" to locals, and their "primitive" social practices such as eating with hands and consuming raw foods as well as their "indolent" and "lazy" natures were believed to be the reason why the Assamese body was the perfect host to the parasite.[2,3] Typologies of racial differentiation were evident as it was widely believed that the Whites were saved from kala azar simply due to their civilizational prowess – they did not eat with their hands but instead used cutlery, and their diets comprised essential fats and protein and were then labeled as nitrogen.[3-5] Giles' findings were ground-breaking in challenging the miasmatic theory of kala azar; the disease was not airborne, nor did it stem from the jungle's noxious fumes.[1] However, by 1896, a report by Leonard Rogers' firmly reclassified kala azar as a form of "malaria cachexia," disproving the proposition by Giles that kala azar was ancylostomiasis.[6]

A year later, Ronald Ross found malaria oocysts in mosquitoes, although Leonard Rogers was quick to counter that in kala azar (which he considered to be malaria cachexia), mosquitoes did not have a role to play.[7,8] Ross, who was then in Bengal, was not the only one studying the link between mosquitoes and malaria in the mid-1890s: physicians and surgeons in Assam were

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also suggesting that mosquitoes played a significant role in malaria transmission – a view not shared by the meticulous Rogers.[6] As Rogers stated, "Dr. Patrick Manson and others have recently suggested that this takes place in ordinary malaria by mosquitoes sucking up the blood from infected persons, and proceeding to die in water, thereby releasing the germs contained in the blood which then multiply. Although Surgeon-Major R. Ross has recently published some interesting experiments on certain changes which the crescent-shaped form of the parasite goes through in the stomach of the mosquito, there is as yet very little evidence in favour of Dr. Manson's ingenious idea and, although I have constantly borne it in mind, I have not met with any facts in the course of my investigation that lend support to it."[6]

Rogers did not consider zoonotic vectors as the agents of malaria transmission and instead opined that soil was the medium which bred germs and kept it active.[6] Although theories of contagion were quickly replacing the beliefs of miasma in the 1890s, Rogers considered the air as infectious, while rejecting the idea that kala azar was borne from the noxious fumes and putrefaction of Assam. Malaria, according to Rogers, originated in the human body due to the inhalation of germs released into the air by moisture evaporating from marshy ground or by groundwater that displaced the air from soil. He explained that these germs entered the lungs, traversing a thin layer to reach the bloodstream, where they thrived. After completing their lifecycle in the human body, they would exit back into the air, aided by the cilia in the air passages.[6] In 1898, after Rogers had published these studies, Ronald Ross arrived in Assam, determined to establish that that the communicability of kala azar was vector-borne, countering the widely held assumption that the local Assamese inhabitants were parasitic.[3-5] He stressed that, "… it had long been obvious that the precise mode of infection by malaria could be ascertained only by the discovery of the life history of the parasites of malaria outside the human body.… some indications had been obtained which went to show that these parasites can undergo development in mosquitoes."[8]

However, Ross was wary of Roger's conclusiveness on kala azar and stated that "Rogers, to whom we are so largely indebted for his work on kala-azar and especially for his boldness in declaring the communicability of paludism, does not, however, I think, afford a very satisfactory explanation of this phenomenon. He attributes it, in the case of kala-azar, to a heightened virulence of the parasites of malaria in that disease, and considers the path of infection to be by way of the lungs. It is little likely, in the case of an animal parasite, that any increment of virulence should suffice to change its mode of propagation and he cites no experiments to prove communication by the lungs. On the whole, I think that the mosquito theory of Manson will suffice to explain the whole epidemiology of malaria, and believe that it may be accepted as established, although many details remain to be discovered."[8]

THE PESTS OF INDIAN LIFE

Besides the *Phlebotomus*, the bed bug was also debated to be a possible vector. In 1907, Leonard Rogers posited that the transmitting vector might be an insect, the *Cimex lectularius,* or "pest of Indian life, the homely bedbug" through studies of acidified blood as the culture medium.[7] This hypothesis was substantiated by the fact that the disease spread quickly in impoverished settlements. The success of segregation and burning down the huts of the coolies in Nagaon, Assam, also lent credibility to this view that the organism of kala azar was inside the house.[7] However, this was just a hypothesis and could not be solely pinned to the bed bug as lice and fleas were also considered putative vectors, and this opinion did not find much popularity in the medical community. In 1909, Charles Donovan wrote that instead of the bedbug, the "*Conorhinus rubrfasciatus*," which is today curiously called the Kissing Bug and is part of the Triatominae family, was the more probable contender.[9-11] It was also called "the mother of bugs" in Madras, where he was stationed. Donovan was keen that the Triatominae was a better contender because unlike the bedbug, it was widespread inside and outside human dwellings, and its habits were much more compatible with disease transmission than the bedbug.[9,10]

Be it the mosquito, the triatomine, or the bed bug, no irrefutable answer was available, and transmission of kala azar infection continued to be hazy. The bed bug theory sprung up again as in 1922 at the Indian Science Congress, when W.S. Patton, a physician and entomologist, held that the bed bug would probably be the only vector responsible, and other vectors like the mosquitoes, fleas, lice, and ticks were ruled out on epidemiological and experimental grounds.[9] The possibility of the vector being the biting midge or the *Culicoide* was discussed, but disputed as the biting midge only occasionally bit humans.[9]

INCHING TOWARD AN ANSWER: THE SANDFLY

It was with the establishment of the Kala Azar Commission that research on vectors reached a new impetus. The Kala Azar Commission, financed by the Indian Research Fund Association and the different Provincial Governments, was constituted early in 1924.

The role of *Phlebotomus* regained prominence in the 1920s and 1930s, as investigations into kala azar transmission were undertaken in three different locations. Robert Knowles led a study at the Calcutta School of Tropical Medicine, H.E. Shortt headed a team in Assam, and W.S. Patton along with Edward Hindle conducted research in China's Shantung Province on behalf of the Royal Society Kala Azar Commission.[12]

Major R. Knowles's team which comprised Dr. L. E. Napier and Assistant Surgeon R. O. A. Smith at the Calcutta School of Tropical Medicine, steered by their previous epidemiological studies to suspect either the *Phlebotomus* (sandfly) or the *Culicoides* (minute midge) as the transmitters of the disease, demonstrated with certainty that the parasites of kala azar developed in the sandfly, *Phlebotomus argentipes*.[12] Field laboratories were established in the Golaghat district of Assam where further studies were carried out. The ancillary inquiries by Knowles, Napier, and Smith from Calcutta were confirmed by the Commission, which found that sandflies fed on the peripheral blood of kala azar cases. In approximately 25% of cases where sandflies fed, the insects developed midgut infections with the flagellate form of the parasite, which became notably heavy after a few days of feeding.[10,13] However, much remained to be done before the sandfly could be definitively identified as the transmitting agent as the method by which the parasite reenters the human body remained unanswered.[10,13]

The Kala Azar Commission's studies were not the first to highlight the potential role of the sandfly in disease transmission. From the 1910s, reports poured in from around the globe: Charles Wenyon reported flagellates in sandflies in Aleppo, Syria in 1912, and in 1914, F.T.E. Mackie found flagellates in the guts of a sandfly identified as *Phlebotomus minutus* that geckos fed on.[10] Almost a decade later in 1925, Edmond Sergent in Algeria found that *Phlebotomus papatasi* sandflies could transmit *Leishmania major*, leading to cutaneous leishmaniasis.[13] In 1925, John Alexander Sinton published his hypothesis linking transmission of kala azar with the *Phlebotomus argetipes* sandfly, although he had shared it with Knowles and colleagues at the Calcutta School of Tropical Medicine as early as 1922.[10,11] Soon, Knowles's team became the first to demonstrate that *Phlebotomus argentipes* sandflies fed on kala azar patients and the *Leishmania donovani* parasite transformed from the amastigote to the promastigote form in the fly's midgut.[13] To replicate these results, Christophers, Shortt, and Baraud reared *P. argentipes* in a lab in Assam and confirmed Knowles's findings with laboratory-bred flies.[9,12,13]

THE COST OF CONCLUSION: THE HUMAN EXPERIMENTS OF THE KALA AZAR

In the first Kala Azar Commission report by S.R. Christophers, H.E. Shortt, P.J. Barron, and L.E. Napier, the vector was identified as one that inhabited domestic dwelling spaces rather than solely the jungle areas. This hypothesis was supported by observations of young children, who rarely left their homes and yet frequently contracted kala azar, contradicting the idea of a jungle-dwelling vector.[10] During the next 2 or 3 years, the Commission focused on experiments of transmission on sandflies and investigated how the parasite was conveyed within the life cycle of the sandfly. Scott approached the question of using human volunteers in his laboratory and in Calcutta, but his request was immediately dismissed as absurd, and he was explicitly forbidden from conducting such trials.[14] However, the response from his superiors in Calcutta also hinted that they might tacitly support the work as long as they were not held accountable.[13] Seizing on this thin straw of hope, Shortt resubmitted his proposal under a new title, "Research with Insects," omitting any mention of human volunteers, and it was eventually approved. However, his colleagues, Barraud and Craighead opposed this study on the grounds of it being unethical and unjustified, but had to reluctantly take part due to Shortt's orders. Moving from the Golaghat district in Northern Assam, where the Kala Azar Commission laboratory was located, to the more accessible town of Gauhati, "volunteers" were brought in from the salubrious hill station capital of Shillong, where the higher altitude ensured the absence of the *Phlebotomus argentipes*. The protocol was carefully designed: "volunteers" who never left Shillong were chosen, they were paid a sum of Rs. 400 per month, and upon infection, urea stibamine was used.[13] The "volunteers" arrived in Gauhati, were exposed to infected sandflies in a sealed room to prevent wild bites, and then were driven back to Shillong for monitoring – all five volunteers became infected, providing conclusive evidence that kala azar could be transmitted by the bites of sandflies. However, Shortt's use of human volunteers faced ethical criticism, with claims that these volunteers might have died due to the experimental infections.[14] **Shortt never** made any mention of these deaths, but fulsomely acknowledged the selfless courage of the volunteers, noting that "We must acknowledge the self-sacrificing spirit of the human volunteers who submitted themselves for experimentation and helped in the final solution of a problem in tropical medicine of many years 'standing.'"[14]

CONCLUSION

The investigation into how kala azar spread and the mystery of its transmission played an instrumental role in shaping research on the disease. The environment was dismissed as the sole source of the contagion from the 1890s, and it was believed that there was more to the disease in terms of human engagement than just the miasmas and noxious air. Human activity became primary to the investigation from the 1890s. Colonial doctors tasked with studying kala azar approached it with imperial arrogance, attributing the presence of the parasite to the race and social customs of the local population. The contagion or germ theory that was rapidly gaining popularity in this period framed soil and air as vectors transmitting kala azar. Only in the 1910s and 1920s did researchers begin investigating alternative vectors, ultimately identifying sandflies as the carriers of *Leishmania donovani*. This discovery, however, was dictated by the racial politics and subjugation as natives were intentionally infected with kala azar as an experiment, with their fates unanswered. The prevalence of kala azar in the dense, tropical lowlands of Assam rather than more salubrious uplands suggests that human encroachment on forested areas, including deforestation and habitation, facilitated entry of the sandfly into human settlements, which was further encouraged by factors like poor sanitation, impoverishment, and an overall lack of awareness accounting for the spread of kala azar.

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Review Article

Controlling kala azar in Assam

Anidrita Saikia

Department of History, Delhi University, New Delhi, Delhi, India

Abstract

By exploring the state responses to kala azar in Assam during the early twentieth century, this review examines how, following the discovery of the parasite, efforts to halt the disease took on new forms, as questions about its communicability remained unresolved. To control the spread of kala azar, segregation policies were implemented as an experiment in the tea plantations and specific districts. In these tea plantations, coercive segregation was enforced, while camps were established in a few villages to relocate the affected families. Segregation proved only a partial solution, leading to the introduction of antimony tartrate injections administered by lower-ranking surgeons and, later, to the establishment of the first Kala Azar Hospital in Nazira.

Keywords: Antimony tartrate injections, Assam, kala azar, parasite discovery

Address for correspondence: Ms. Anidrita Saikia, Department of History, Delhi University, New Delhi, Delhi 110021, India.
E-mail: rukmisaikia0307@gmail.com
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CONTROLLING CONTAGION: SEGREGATION IN ASSAM

The discovery of the kala azar's parasite in 1904 elicited little response from the colonial regime regarding the control of its transmission, leaving officials with uncertainty. As a result, kala azar continued to spread, infect, and kill, prompting the introduction of new control measures. By 1913, provincial surveys of kala azar were established and became the standard practice, with the intent to gather data on the location of the patients and identify the villages and stations with the highest infection rates.[1] By the turn of the nineteenth century in Assam, kala azar had infected a large portion of the population residing in the Brahmaputra Valley, but had not yet spread to the uplands of Northern Assam. The depopulation and the high mortalities of the station towns in the Brahmaputra Valley, especially Nowgong, raised concerns of the disease's impending approach to the North.[2]

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To stem the spread of the disease, the Epidemic Diseases Act of 1897 was enforced to restrict the movement of people as a containment measure. The Act laid that during or under the threat of an epidemic, when ordinary provisions of the law were inadequate, the State could implement necessary measures and issue public notices to establish temporary regulations to forestall the disease.[3] Segregation and movement control were merely two among the array of powers given to the State under the Act. The Act allowed officials to inspect people anywhere, stop public congregations, destroy property and houses, and ensure the coerced hospitalization of suspected cases, even without definitive medical proof.

COERCION IN THE TEA PLANTATIONS

Isolation, segregation, quarantine, and relocation are not unknown to contagion, but enforcing segregation and isolation proved challenging to implement, especially in

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closely populated settlements like the coolie dwellings in the tea plantations of the Brahmaputra Valley. The tea gardens were unique sites of control where the tea planter's interpretation of the law was final. Each garden frequently employed its own methods to respond to the disease. In one tea garden of Nowgong, when cases escalated and quinine failed as a medical aid, "new lines were built in winter as a 'vigorous measure to eradicate the disease' where all the healthy, non-infected coolies were transferred. The huts where the infected coolies lived were 'destroyed by fire."[3] This was the most frequently employed strategy, endorsed by the kala azar expert, Leonard Rogers, and adopted by doctors and civil surgeons; the establishment of a new coolie line with new huts constructed at a distance from the previously infected lines was encouraged.[3] In September 1898, Rogers reported that Dr. McIntyre of the Mangaldai district wrote to him that "the moving of the recently-infected coolie lines on a garden he was in charge of, proved a success [Figure 1]."[4] The burning and destruction of an infected hut was thought to be crucial to curtail infection, much to the ire of the coolie workers.

In 1898, Dr. Dodds Price, the civil surgeon of Nowgong, reported the instance of segregation and relocation in a tea garden of Solona. "In the Old Solona lines (the infected ones), there were 240 working souls, of whom I found I46 either suffering from kala-azar.… Of these we canceled the contracts of I7 adults and cleared them out; the remainder we sent to the infected lines at Rangamati. The 94 healthy people we drafted into the new lines at Old Solonia...without a single kala azar case among them. To begin with we had one or two doubtful cases among the old-line coolies, but these were immediately sent over to the camp at Rangamati. By acting promptly, we thus kept the new lines free from kala-azar, and now 15 months since their completion there has been no death from the disease. Now, as to the 60 coolies living in the lower lines, these, being old residents and free people, could not be interfered with, and refused to be saved from themselves. Kala-azar has spread to them, and accounted for 6 deaths up to the end of the year."[4] Thus, relocation and segregation, essential to containing an epidemic, were implemented with success in the tea gardens, but under the iron fist of coercion and control. Prior scholarship has detailed the abject state of labor in the gardens, and the coolie was described in the planter's lexicon as "primitive," "jungly," "slothful," "scoundrel," "absconder," and so on, reinforcing the colonial rhetoric that the coolies "die very easily."[5]

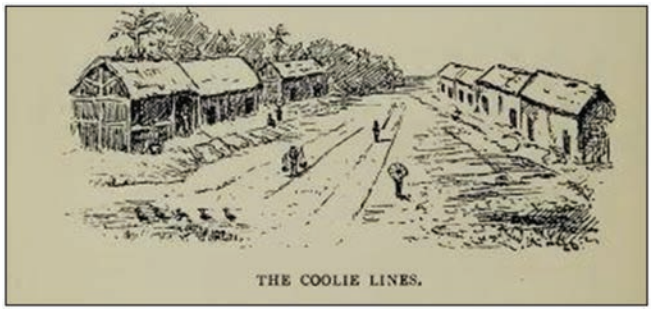

Figure 1: A sketch depicting the coolie lines in George Barker's Tea Planter's Life in Assam. Courtesy: Barker, George. Tea Planter's Life in Assam. Calcutta: Thacker, Spinck and Co., 1884, 134

RELOCATIONS IN THE VILLAGES

Among the Assamese villages, kala azar containment measures took a somewhat different trajectory. How was the disease to be contained when routes of travel and communication were open? Golaghat serves as an illustrative case study as it was the first subdivision that enforced evacuation rules based on segregation. In 1911, a few villages in Golaghat harbored active cases, raising concern among officials that kala azar could spread to uninfected Upper Assam.[2]

Earlier efforts at containment and segregation yielded varying degrees of success. The disease originated in Golaghat in a village called Khumtai, where nine families resided.[2] In 1911, these families were forcibly relocated to a nearby village, where they were reported to be disease-free. Despite this, the disease remained active in Khumtai. Within a single household of six members which did not relocate, only two managed to survive following infection with kala azar, while the others succumbed.[2] The official perspective was that relocation would halt the spread of infection and prevent contamination of the surrounding environment. The policy did not work: between 1911 and 1914, fresh cases continued to emerge even after relocation.[2] Despite the rapid rate of transmission and the severity of the colonial officials were still very much concerned. The Sanitary Commissioner writes that in Golaghat, "The people appear to recognize the value of the measures, and are grateful to Government for carrying them out, provided no new and separate foci of the disease appear, it is quite possible that the disease may be extinct in this subdivision in a few years' time, if these measures of control are continued."[2]

Historically, segregation has been often enforced to contain the spread of the contagion. Although the medical reasoning was understandable, concerns driven by anxieties around race, class, and caste persisted. Under the guise of "saving native lives" hid coercive and violent policies of razing down homes, displacing families to camps and hospitals, and so on. Naturally, this resulted in resistance

and opposition.[6] That the "people appear to recognize the value" might have held true only for a small section of the population. In the tea gardens, infected coolie families who were removed to new huts were far from grateful. T.C. McCombie Young reports that in a Golaghat tea estate in 1917, "108 cases of kala azar had been recognised, 38 deaths had occurred, but removal operations had commenced.…the manager had succeeded in providing 104 houses for uninfected families and 34 families…the labourers, being disturbed by these removal operations, threatened to abscond. Government was faced with the risk of dissemination of the disease throughout the country by these absconders on other tea estates and the management was faced with the depletion of a labour force."[7]

WHEN RELOCATIONS FAILED: THE PROBLEMS OF SEGREGATION AND THE INTRODUCTION OF ANTIMONY VACCINATIONS

The process of removal and resettlement was dependent on the principles of segregation. Government regulations regarding segregation were established in 1917 and put into practice, particularly in the Sibsagar district where 33 out of 58 villages were placed under segregation [Figure 2].[2] Implementing segregation through resettlement proved complex as separating the infected from the non-infected presented challenges that authorities were reluctant to address. Logistical difficulties too abounded, as Young complained of how such relocations involved labor and expense and that homestead lands nearby were difficult to find.[7] Each family was to be paid a hefty sum of around Rs. 300 for relocation, and despite official efforts, new cases still emerged.[7] It was obvious that these measures did little to provide relief and failed to halt transmission within the community, and relocation even held the inadvertent possibility of accelerating kala azar's spread to previously unaffected areas.

Figure 2: "House built by government for segregated family in the Sibsagar district." A photograph showing the new huts built where the segregated families were put up. Courtesy: McCombie Young TC, Kala Azar in Assam: An Account of the Preventive Operations 1910 to 1923 and Notes on the Epidemiology of the Disease in Assam and India (London: H.K. Lewis).1924

However, things slowly started to change as in 1920, the Sanitary Commissioner noted that segregation "was not found possible of application on a large scale. Segregation is undoubtedly of value in preventing spread if applied with sufficient thoroughness and carefully supervised and enforced but provides no relief for the actual sufferers from the disease and successive crops of cases amongst the segregated are frequent."[8] In some infected villages, where relocation was not enforced, treatment with emetic tartar was initiated and demonstrated encouraging result.[7] As the mode of transmission of kala azar had not yet been discovered, segregation continued to be encouraged. Regarding transmission, which remained the "great unsolved mystery," evacuation was seen as a promising measure to prevent future infection and was the only preventative measure.[7] This practice began in 1912 in Naharani Mouza, Golaghat, with the removal of infected families and patients. The infected houses, typically mud-plastered, grass-thatched huts with wooden and bamboo frameworks, were burned down along with any less valuable property. Compensation was provided for the destruction, and valuable items were disinfected.[7]

LOCAL RESPONSES

Unlike segregation camps set up in Bombay and Karachi after the onset of the plague, relocation in Assam was usually to a nearby site.[9,10] New houses were initially built within 10–20 yards of the previous site, a practice that failed. In an attempt to separate the infected individual from the non-infected family, a separate shed was built nearby, which failed since the sick were usually children who could not live in isolation.[3,7] Unlike the protests across different parts of India in response to compulsory segregation, local responses from the Brahmaputra Valley (except for the tea plantations) to kala azar segregation measures were met with little resistance. Was this a response from a subjugated population quietened down by systematic terror, or was the practice already familiar with the natives? Or was it simply because the family would get a handsome sum in exchange for quiet relocation? Young remarked that the locals were cooperative in these relocations despite the intrusive nature of the project, which he attributed to the deep-seated fear of kala azar among the Assamese.[7]

Segregation and evacuation, now mandated as compulsory by the states in the regions that it was enforced in, was a practice earlier used by the Garos in the Garo Hills. In 1897, Rogers wrote that the Garos were traditionally believed to take affected individuals into the jungle, where they would drug them into unconsciousness, set fire to the temporary huts they were placed in, and burn them to death.[3] The visage of the Garo's burning hut would come to be cemented in the imaginations of kala azar, with British Parliamentary papers stating the "primitive" Garo villagers "intoxicated the sick and then 'set light' to their houses, incinerating the occupants."[11] Such a practice comfortably aligned with the labels of "savage," "rude," and "primitive" that ethnographer officials had plastered on the community but erased the desperation of a terrified tribe to a disease that they knew nothing about.

Women, unsurprisingly, bore the brunt of the native response to the epidemic. In medical inspections that were carried out as part of segregation projects, many women did not want their bodies to be touched and prodded.[7] When writing about a sick Garo woman, G.M. Giles observes that "No one would come near her, she was alone in a miserable hut, which was tumbling down. Her food and drink were handed in at the door, none of the villagers would touch her: even her husband had taken another house in the village, and the children called out to the Hospital-Assistant and myself, and warned us not to touch her. When they found we had, they immediately called out 'you will get Kala-azar'."[12]

In the relocated villages of the Brahmaputra Valley during the 1910s, there were three modes of segregation. An "infected camp" where infected families and individuals were kept in different huts; a "contact camp" of a few huts where the neighbors of the afflicted as well as anyone suspected to be infected were placed; and a "healthy camp" where the rest of the non-infected villagers were kept.[7] The segregated contacts in the infected camp would be closely watched by an official on duty or a headman. The sale of fowl between the "infected" and "noninfected" villages was prohibited, and young women were prohibited from marrying between the camps unless an official documented her health in a certificate[7] Those in the infected and contact camps had their names on a roll which was checked by officials regularly.

TRAVELING SURGEONS AND A LAKESIDE HOSPITAL: THE SHIFT TOWARD PREVENTION AND CURE

To ensure effective observation and management, a set of new recommendations was established in 1913. From

Figure 3: The first kala azar hospital of Brahmaputra Valley at Nazira, Assam. Courtesy: McCombie Young TC, Kala Azar in Assam: An Account of the Preventive Operations 1910 to 1923 and Notes on the Epidemiology of the Disease in Assam and India (London: H.K. Lewis).1924

1914, six sub-assistant surgeons, equipped with mobile dispensaries, were to be dispatched on tours throughout the endemic regions, treating infected individuals, monitoring the disease's progression, and ensuring segregation.[13] Racial hierarchies were firmly entrenched within the medical bureaucracy, and the Sub-Assistant Surgeons (or the SAS) were native assistants.[14]

From 1919, a shift from segregation to preventative medicine was observed, and hospitals solely for the treatment of kala azar were established. The first such hospital in the Brahmaputra Valley was in Nazira in Sibsagar District [Figure 3]. Situated in the village of Borkula in Nazira, the acquisition of land of 6.8 acres was completed in September 1918, and the hospital was started in 1919, as Figures 2 and 3 illustrate. The hospital, an unfamiliar concept to the nearby locals, was initially seen with suspicion, but patients soon started filling in, and it became quite popular.[7]

In the villages, sub-assistant surgeons were allotted a certain area within a rural circle where a temporary kala azar dispensary was established. This consisted of a small house and a three-room shed which contained all the appliances and equipment needed for treatment. The sub-assistant surgeon's equipment comprising syringes, burners, and trays were designed to be portable and functional. The kala azar surgeon would arrive at a village, complete his duty of vaccinations, pack up, and

move to the next village Although distilled water was crucial, it was not provided. Instead, the officer had to carry a Berekefield traveler's pump which would filter and sterilize the local water. The metal needles were reused for multiple emetic tartar vaccinations; after each use, they were cleaned and sterilized with Vaseline at 160 degrees Fahrenheit in a Kapadia sterilizer.[7] All of this was carried in the "kala azar pannier," a wooden box created in 1920 by Major Taylor during his duty related to kala azar.[7]

CONCLUSION

As the only available preventive measure, segregation was guided by the unanswered questions about transmission and concerns about site-based infection that led to the relocation of infected households. The tea plantations became the first experimental grounds of this segregation where exploited coolies were forcibly moved and their huts burnt. Assamese villagers faced relocations in the form of camps and household segregations of a village. But as new cases continued to emerge among segregated families, it became clear that segregation was not a sustainable solution and could not be implemented on a wide-scale basis. The success and adoption of emetic tartar shifted the focus from addressing the attempt to control kala azar's transmission to diagnostic cure, culminating in the establishment of the region's first kala azar hospital.

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Review Article

Early treatment modalities and responses to Kala-azar in the 1920s in Eastern India

Anidrita Saikia, Sudip Chatterjee[1]

Department of History, Delhi University, New Delhi, Delhi, [1]Park Clinic, Kolkata, West Bengal, India

Abstract

From the 1920s, there was a shift in the treatment modalities with the presence of small dispensaries in rural areas that still partially relied on quinine, along with establishment of specialized kala-azar treatment centers. The initial use of sodium antimony tartrate injections sparked concern as side effects were being reported, inciting a search for alternative treatments. By drawing on diverse voices, including civil surgeons, health officers, and municipal leaders, a multifaceted view of kala-azar treatment is presented in this review.

Keywords: 1920s, alternative treatments, kala azar, public health, treatment modalities

Address for correspondence: Ms. Anidrita Saikia, Department of History, Delhi University, New Delhi, Delhi 110021, India.
E-mail: rukmisaikia0307@gmail.com
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KALA-AZAR CENTERS IN RURAL BENGAL

By the mid-1920s, Bengal and Eastern India, especially Assam, had established a well-structured response for the control and prevention of kala-azar. Kala-azar treatment centers were established in numerous districts across various provinces in Bengal. However, these centers faced challenges due to an insufficient medical workforce and a burgeoning population, with few doctors to attend to the increasing number of patients.[1] While Calcutta could boast of efficient public hospitals, resources and access were hardly favorable in the rural areas and the interior districts. In Mymensingh, Dr. Bidhuranjan Chakravarty, the District Health Officer, observed that kala-azar had not reached epidemic levels till after 1915, with cases only appearing around 1917, attributing it to migrant laborers from the villages that bordered Assam, who went there to work and brought the disease back to Mymensingh.[2] This was a popular opinion among health officers of the easternmost districts: the District Health Officer of Tipperah, Dr. H. Mukherjee reported that kala-azar cases appeared in the villages of Tipperah district of Bengal only from 1915 where migration was frequent, and became rampant by 1921.[2]

Combating an epidemic in these rural districts proved difficult. Dr. Jahar Lal Dey, the District Health Officer of Jessore, was dismayed at how public ignorance posed a significant barrier.[2] Despite the presence of kala-azar treatment centers, attendance often declined because village "quacks" would visit homes of the villagers, offering quick fixes for minimal fees – an approach that many illiterate patients preferred over waiting for prolonged hours at the official centers. Dey wrote that treatment by these "quacks" was detrimental to public health and were irrational, careless, and often dangerous – although these self-guised "healers" had access to Western medicines, they administered them without regulation, giving malaria

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patients dangerously high doses of antimony.[2] In districts with a predominantly conservative Muslim population, women's access to treatment was severely restricted, usually by male family members. In Mymensingh, for instance, men often prohibited women from visiting treatment centers, and male doctors were usually not permitted to enter their homes.[2] Some kala-azar centers tried implementing a purdah system that allowed Muslim women to receive injections, while protecting their modesty, an approach that proved unsuccessful. The absence of female doctors and medical staff was sorely felt, and the fund crunch was evident.[2]

The concerns over kala-azar among the residents of Calcutta were starkly different from those in the rural villages, and the disparity in the concern and response between the educated, urban elites of the city and officials from the rural areas made the unevenness of healthcare distribution evident. S.N. Mallick from Hooghly stated that combating kala-azar requires a multifaceted approach involving various stakeholders of both the urban and rural. He reported that in the kala-azar treatment center in Hooghly, patients traveled around 14 miles to receive care in the kala-azar center, which reported 637 patients in October 1924. The wealthier elites that included zamindars and upper-caste landed families frequently moved to Calcutta, where they had better access to medical treatment and improved sanitation.[2] Mallick thundered that, "We think that the only grievance in this country is the British Indian Constitution… We are criminally guilty of neglecting sanitation and other vital issues over the years. Those without financial means are forced to remain in villages, while the educated and capable, especially women, are drawn to the allure of Calcutta life, with its electricity and piped water. Consequently, villages are being abandoned." Mallick highlighted that kala-azar was only one epidemic challenge; chronic malaria left the population weak and the youths became invalids and were unable to address local sanitation norms; villages had to thus depend upon migrant labor from Chota Nagpur to clear jungles and stagnant water tanks. He argued for a collective grassroots approach to address these issues, emphasizing the need for aid and funding to support the community.[2]

Under Malaria's shadow

Much before the epidemic advent of kala-azar, malaria was rampant. It is unsurprising that the febrile nature of kala-azar was always closely associated with malaria, being called "the offspring of malaria."[1] Kala-azar cases were often treated at malaria centers and civil dispensaries, where quinine remained the primary treatment despite the availability of antimony, although municipal responses were restricted. The chairman of the North Dum Dum municipality, Babu Sailaj Lal Chatterjee, noted that efforts at the local level were restricted as charitable dispensaries had limited funding.[1] The close association of malaria with kala-azar also made early diagnosis difficult, although attitudes started changing: when a kala-azar center was opened in a district's interior, Dr. A.N. Mitra observed that the doctor administered antimony injections to kala-azar patients, while those with malaria received quinine.[1] Antimony administration brought relief to many kala-azar patients, and word of this effective treatment spread rapidly through nearby villages, resulting in patients in the disease's sub-acute phase arriving in the center from far away. Dr. Mitra emphasized that effective centers for kala-azar treatment required strong community engagement, consistent staffing, accessibility, and sufficient time for residents to become aware of their services.[1]

Yet, well into 1924, an apathetic attitude in these treatment centers pervaded. Dr. Rai Gopal Chandra Chatterjee wrote that, "Even now the authorities in charge of charitable dispensaries, in the interior of the districts are not willing to change the arrangements made in the pre antimony days, they are being managed like in the old times - palpable kala-azar cases being designated as malaria cachexia and treated with stock spleen mixtures. They are however, slowly being roused to the injection centers. Even in Calcutta, the doctors in charge of the outdoor charitable dispensaries, objected to the method of introduction of the injection of kala-azar cases by antimony [because] they have no spare time for giving kala-azar injections."[1] Besides the evident inadequate medical workforce, Dr. Chatterjee argued that the only effective way to address this challenge was to establish more kala-azar centers.

While hospitals in Calcutta had access to appliances and aids, civil dispensaries in district and municipal centers had to rely primarily on clinical symptoms and aldehyde tests for diagnosis of kala-azar. Dr. Rakhal Das Roy, the District Health Officer of Malda, expressed concern over the limitations in diagnosing kala-azar accurately. Initially, Malda's district headquarters lacked even a microscope, forcing the doctor's inspection on observable clinical symptoms of the body.[2] It was only toward the end of 1924 that microscopes and other necessary equipment became available, allowing staff to examine blood for *Leishmania donovani* (LD) bodies. By 1925, Malda's *sadr* headquarters was equipped with a laboratory, and diagnosis using aldehyde and formaldehyde tests became common practice. This improved access to diagnostic tools marked a significant advancement in kala-azar detection in the region.[2]

Antimony as treatment, arsenic as an approach

Antimony, formulated and used as emetic tartar in the early seventeenth century by German alchemist Adrian de Mysinch, gained popularity for the treatment of plague and other ailments.[3] During this period, metallic antimony cups were believed to have healing properties and became popular. When wine was left in these cups, it dissolved antimony compounds, but unchecked use led to illnesses and deaths, especially among medieval monks. This may have influenced the term "antimony" (which derives from the Latin word "stibium"), reflecting its harmful effects. These deaths soon brought a ban on antimonial remedies in medieval Europe.[4]

Only in the 1910s did antimony re-emerge as a treatment for tropical diseases, following Thomson and Cushny's successful studies of antimony to combat trypanosomiasis in rats, which eventually paved the way for human use.[5] The use of antimony to treat kala-azar in India began in 1915 after Sir Leonard Rogers and Dr. Muir observed the successful outcomes reported by Caronia and Di Cristina, who treated Mediterranean leishmaniasis with intravenous injections of emetic tartar in Italy.[3] In Bengal, a 2% sodium antimony tartrate solution was used across all treatment centers. Adult patients were given a beginner's dose of 0.5 cc, while children under 10 received 0.25 cc. The number of injections required for successful cure ranged from a minimum of 7 to a maximum of 91, with doses administered bi-weekly.[2]

Antimony was not the only diagnostic medicine. A tonic pill was also prescribed containing 324 mg of quinine sulfate, 5.4 mg of ferric arsenate, 8.1 mg of aloin, and 0.65 mg of strychnine hydrochloride, taken once or twice daily based on symptom severity. For children, a powder composed of 16.2 mg of emetic tartar, 32.4 mg of tannic acid, and a saccharated lactose base was administered orally in the mornings.[2] This treatment proved effective, with over 600 cases reportedly cured. Before the introduction of emetic tartar, quinine was administered and was a popular means of recourse till 1915 for kala-azar, and even after antimony's discovery, the locals were given quinine pills as a preventative measure during the malaria season.[2] Leonard Rogers had written that success in curing malaria using quinine was only moderate, with about 75% cure – but medical opinions countered that instead of large doses administered orally, there were more chances of success if smaller doses of quinine were given intravenously. In 1911, Muir's trials were recorded, where he injected two to six grains of quinine sulfate in acid solution through intramuscular injection, after previously inserting 5 minims of a 2%, which resulted in severe pain, which was countered with codeine. According to him, if cases were identified within the first six months and treatment was continued, it seldom failed.[6]

Experiments with atoxyl and iodine

Other approaches to treatment included sodium arsenic or atoxyl; S.L. Sarkar's studies showed promising effects of atoxyl against some trypanosomiasis cases, despite having no impact on trypanosomes in laboratory cultures.[6] This was not the first time that atoxyl was used in treating kala-azar; in 1907, Dr. Andrew M'Caig had tentatively administered atoxyl to a patient in Kalimpong and was surprised at its efficacy. Sarkar's explorations of atoxyl was based on the studies of Paul Ehrlich who prepared many derivatives of atoxyl and found that compound 606 (Salvarsan) was the most effective to cure syphilis in rabbits, leading to the breakthrough way for the use of Salvarsan in syphilis from 1910.[7] Sarkar documented the case of a 7-year-old boy with kala-azar suffering from spleen enlargement and recurring fevers, who was injected with half a grain of soamin on every alternate day. After a few days of injections, the boy began showing signs of arsenical poisoning: diarrhea, frequent urination with traces of albumin (which had not been present before), eye congestion with pronounced photophobia that prevented him from opening his eyes, and a burning sensation over his body.[6] The soamin injections were stopped, and a dose of *Staphylococci* vaccine was administered. Sarkar was vague and reluctant in his explanations, but asserted that the *Staphylococci* vaccine was not only effective in treating leukopenia but also showed significant metabolic effects.[6] For instance, in cases of diabetic carbuncles usually caused by staphylococcal infection, the vaccine could reduce sugar levels in the urine, and Sarkar was hopeful that it might alleviate atoxyl poisoning. The toxic symptoms gradually subsided, and the patient improved, recovering from his fever within a week. Two years later, he remained healthy, with no eye issues such as optic neuritis or atrophy.[6] Sarkar believed that an extra dose of atoxyl byproducts had developed in the patient's body, causing mild toxicity while eradicating the parasites. This theory supported Ehrlich's hypothesis that atoxyl's effects came from a slowly forming byproduct that was toxic to both parasites and (to a lesser degree) the host, and the gradual elimination of parasites minimized harm to the patient.[6]

In 1915, Rogers detected that the proven effectiveness of certain arsenical treatments for human and animal trypanosomiasis prompted their possible effectiveness against the *Leishmania donovani* parasite.[8] The presence of anemia and changes in bone marrow further supported the use of arsenic, which could stimulate an increase

in leukocyte levels in the blood; however, the oral administration of arsenical solutions often led to diarrhea. Although Salvarsan's subcutaneous injections generated optimism for treating kala-azar in the early trials of 1911, results were far from successful.[1] A patient with double remittent fever saw a gradual return to normal temperature after a 0.3-g dose, and another patient with mild fever showed no improvement, with parasites still present after 18 days. In other cases, a patient gained weight after two 0.3-g injections but left the hospital with lingering fever; one developed phlebitis post-injection, another had fatal cerebral symptoms, and a third succumbed to complications from a gluteal abscess following an injection. Overall, salvarsan use in kala-azar treatment presented significant risks, particularly in emaciated patients with low vitality, and did not demonstrate specific therapeutic benefits for the disease.[1]

Iodine was also considered after its successful application in treating plague. Injections of potassium iodide solutions were administered to kala-azar patients in increasing doses starting from three minims up to eight minims and administered every 2 days. However, no clinical changes were observed, and iodine had no effect. Finally, sodium nucleate, which was thought to stimulate white blood cell production, was tested for its potential to boost leukocyte counts, using 0.1- to 0.4-gram doses in a 5% sterile solution. Though carefully sterilized to prevent decomposition, injections in the abdominal wall caused intense pain that demanded morphine use for sleep and fomentations to ease swelling. Although no abscesses or serious side effects developed, but the results were disappointing, with little-to-no sustained increase in leukocyte counts.[8]

U.N. Brahmachari's urea stibamine: A breakthrough

Nothing, however, was as groundbreaking as the discovery of urea stibamine as a cure for kala-azar. Dr. Upendranath Brahmachari, today hailed as "forgotten genius" is central to this breakthrough and the larger history of kala-azar.[9] As emetic tartar's side effects came to be more pronounced, Brahmachari began exploring alternatives to organic antimonials. The success of atoxyl led him create a compound that was both effective and free of the painful side effects of emetic tartar and arsenic treatments.[10] Drawing on research about the beneficial effects of urea salts, Brahmachari developed a new antimony compound by combining para-aminophenyl stibnic acid with urea which he named "urea stibamine" in 1920[11-13] He began administering it to patients at the Campbell Medical Hospital, and the results exceeded expectations. By 1923, multiple cases at the Calcutta Medical College Hospitals showed that the disease was effectively cured in just 3 weeks with a 1.5-g injection of urea stibamine, leading to its enthusiastic adoption as a treatment for kala-azar.[14]

The use of urea stibamine was started on an experimental scale in 1925, and the successful results led to mass adoption from 1928. The Director of Public Health of Assam, Murison, highlighted that emetic tartar caused severe side effects, but sodium antimonyl tartrate, a refined version of emetic tartar that was believed to be less toxic, led to a more prolonged treatment duration.[13] Despite the Epidemic Disease Act requiring the full course of treatment, many patients discontinued visits to treatment centers. The tropical climate of Assam led to storage issues with solutions of potassium and antimonyl tartrate, where the salts quickly decomposed, and the humid weather of Calcutta affected the rubber-capped flasks of the solutions, aggravated by the repeated puncturing of rubber caps while administering doses, which led to bacterial contamination.[13] Urea stibamine proved especially valuable in the rural and remote areas of Assam, where treatment centers were scarce and lacked indoor facilities. It was clearly popular, and the statistics were promising: Between 1925 and 1936, Assam saw a dramatic decline in kala-azar cases and related deaths. The number of deaths decreased from 6365 in 1925 to just 763 by 1936, while reported cases decreased from 60,940 to 10,587 over the same period.[13] [Figure 1]

Antimony fast cases and side effects

Although the success of urea stibamine had been remarkable, it is worthwhile to bring into considerations the complications and side effects that antimony led to and why an alternative was necessary. By the mid-1920s, antimony, the primary treatment option for kala-azar, had become a topic of considerable debate. While sodium antimony tartrate generally yielded positive results, medical concerns had been mounting over its complications. Reports indicated instances of antimony-resistant cases, which Dr. Nani Lal Ghosh of the Central Cooperative Anti-Malaria Society termed as "antimony-fast kala-azar cases."[2] Unlike patients with complications of the kidney, lung, or intestinal issues, where antimony often led to worsening symptoms or death, antimony-fast cases involved patients who tolerated the treatment but showed no improvement, with negligible effects on spleen sizes. Similarly, in Malda, Dr. Rakhal Das Roy wrote that despite the widespread use of antimony for kala-azar treatment, results were not always successful. For instance, a 5-year-old girl suffering from kala-azar received 50 antimony injections with minimal improvement.[2] She was then switched to 13 injections of urea stibamine, but this also showed little effect. Similarly, a 15-year-old boy initially misdiagnosed with malaria was later confirmed to have had kala-azar received 32 sodium

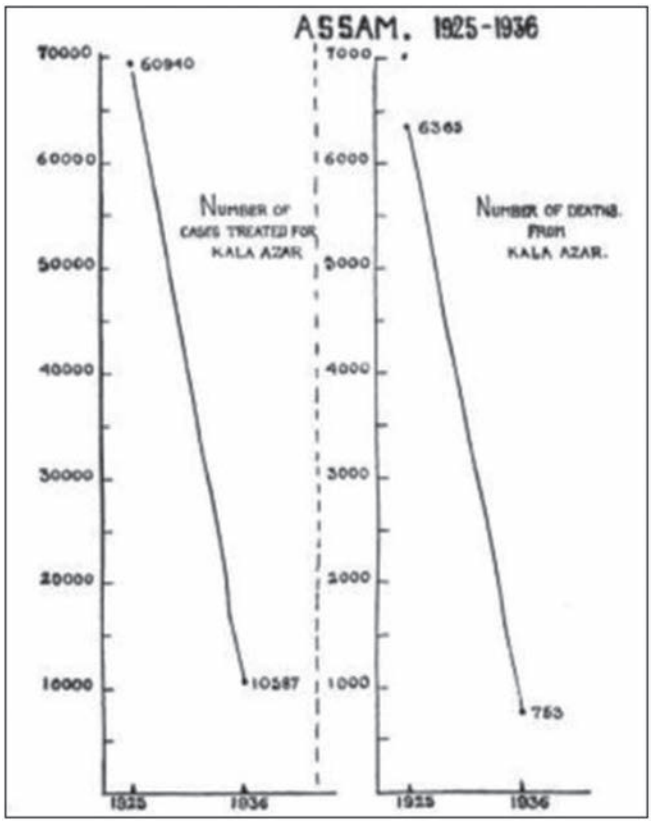

Figure 1: Statistics showing the number of treated cases and number of deaths from kala-azar in Assam from 1925 to 1936. Courtesy: Brahmachari, U. (1942). Note on the history of the treatment of kala-azar with urea-stibamine*. In Gleanings from My Research (Vol. 2). University of Calcutta, p. 795

antimony injections and 16 urea stibamine injections, yet his condition showed no improvement.[2] Another case involved a 24-year-old man who sought treatment at Malda's Sadr Hospital in 1924 and was administered 39 injections of sodium antimony, followed by 11 bi-weekly doses of urea stibamine, and finally 10 injections of Von Heden, all without any positive response.

The side effects of sodium antimony solutions for kala-azar treatment were well documented as well, with symptoms such as coughing, joint pains, skin eruptions, and headaches noted by Muir and Napier and observed in Malda by Rakhal Das Roy. However, an especially concerning case involved a 14-year-old boy who received 61 sodium antimony injections, totaling 40 grains (2.6 g), for kala-azar.[2] Before his illness, the boy was known for his sharp intellect and was one of the brightest students in his school. Yet, within 3 months of completing treatment, his teachers and family noticed a dramatic decline in his cognitive abilities, a dramatic decline in his cognitive abilities; he became unusually dull and eventually abandoned studies. The civil surgeon of Sylhet attributed this as a side effect of antimony. Alarmed, Dr. Roy enquired further in Malda, visiting kala-azar treatment centers and schools in the district, and uncovered over a dozen similar cases, where children exhibited signs of mental impairment following antimony treatment.[2] Education in Malda was still quite underdeveloped, and children's enrolment in schools was far from the norm. As a result, cases of mental retardation could only be identified among the children who attended school, the larger population of children working as agriculturists or as informal labour was likely overlooked. Important to note the informal labour since agriculture was the dominant mode of production, it subsisted with other economies as well. Dr. Roy suspected that many youths outside the school system, who were not monitored or recorded, probably suffered from similar cognitive impairments, but remained undetected. Such mental side effects of antimony treatment were also noted by Sudhir Bose, Assistant Secretary of the Central Cooperative Anti-Malaria Society, who observed that "rare instances of insanity have been noticed."[2] Bose documented four such cases where symptoms appeared as patients neared the end of their antimony injection course or shortly after being discharged as cured, although patients were eventually considered to have recovered later. Cognitive impairment as a side effect was not new. In 1923, Chopra wrote that cerebral symptoms could arise with prolonged injections.[3] Patients often experienced depression, with some developing persistent migraines during treatment. When headaches did not respond to sodium salicylate and sodium bicarbonate, the injections were paused. In rare instances, loss of consciousness followed an injection. Bose also highlighted reports of night blindness, a less common but recurring complaint across treatment centers, with at least one case reported in nearly every facility. The standard response to night blindness was to reduce the dose or pause treatment temporarily. Joint pain was frequently reported and quite common, and lowering the dose typically alleviated the pain along with codeine.[3]

In Chopra's study of with antimony solutions in rabbits, he compared the tissue effects of different antimony compounds. Potassium antimony tartrate caused significant edema, hyperemia, and muscle necrosis. Sodium antimony tartrate led to less severe edema and hemorrhaging with muscle friability. Stibacetin caused minimal edema and hyperemia, while stibamine showed only slight local effects without edema or hyperemia.[3] Chopra writes that "Dr. U. N. Brahmachari has pointed out that rigors and rise of temperature after the first injection are not uncommon in kala-azar cases. It has been suggested that this may be due to destruction of the parasites. We have observed a reaction occurring later in the course of treatment which in some ways is rather suggestive of protein shock. The

patient becomes somewhat collapsed, subcutaneous and submucous haemorrhages occur in various parts of the body and an erythematous rash appears on the body and limbs. We have seen this occur in a few cases of kala-azar and have always noticed that it was accompanied by a rapid reduction in the size of the spleen and followed by a general and very decided improvement in the condition of the patient."[3]

A Cure Too Late: Sukumar Roy

One notable, unfortunate victim of kala-azar was Sukumar Ray, the second of Upendrakishore Ray's six children [Figure 2A]. He was born on October 30, 1887 and his artistic talent emerged in his college days. He wrote plays and often acted in them. His affectionate irreverent wit kept the audience in splits. *Lakshmaner Shaktishel* is one such play, a spoof on the Ramayan. In 1911, he won the Guruprasanna Ghosh scholarship to study photography and printing technology in England. During his stay in England, he met Rabindranath Tagore, a friend of his father's, who had arrived in London in 1912 with the manuscript of *Gitanjali*.

Sukumar returned to India in 1913 to join his father at his newly opened office, U. Ray and Sons, Printers and Publishers. Upendrakishore had started and edited a children's magazine *Sandesh*. For the magazine, Sukumar wrote little stories for children which could be read aloud, travel stories, animal stories and short biographies. After his father's death in 1915, the mantle fell on Sukumar and his brother to run the press and Sandesh. This was the start of the book *Abol Tabol*, his best known work. Here were caricatures of real people whom he had known or met, descriptions of contemporary incidents and so on. There was the doctor who could tackle any ailment with his intuition and basic implements. He had the cure for cholera or dengue, Kala azar or 'pala zar' (presumably malaria), with one well aimed hit of his hammer. Ominously for Sukumar, kala-azar had a mortality rate of 90% with no cure in sight at that time.

In 1919, Sukumar married Suprabha Das, also from a prominent Brahmo family; they had a son in 1921 and soon after the birth of their son, Satyajit, Sukumar fell ill. Notwithstanding his bad health, he continued to write and with the help of his brother, kept the press going. Sukumar was getting weaker despite the careful treatment of his doctor and close friend, Dwijendranath Maitra and survived another two years. He died on September 10, 1923, leaving the young Satyajit fatherless. Satyajit later did an ink drawing of his father [Figure 2B], perhaps from an old photograph. At this time, there was no marketed

Figure 2: (A) Photograph of Sukumar Ray. (B) Sketch of Sukumar Ray by Satyajit Ray.

cure, and ironically Brahmachari had just discovered urea stibamine, which could perhaps have saved Sukumar's life. Like Halley's Comet, of which he had written when it had appeared just before he left for England, he came briefly to light up the sky and went away into space, but left a legacy of versatile brilliance which is manifested in his inheritors in very individual ways.

CONCLUSION

The responses to kala-azar were constantly mediating between the colonial state, physicians, medical researchers, and officials. Kala-azar treatment centers were established to provide diagnosis and cure across the province. While urban centers such as Calcutta had better medical infrastructure and resources, rural areas in Bengal struggled with limited medical staff, insufficient funding, a lack of public awareness, and an increasing population bound to conservative social norms. The evolving treatments and diagnostic approaches demonstrated variability in efficacy: antimony showed toxic side effects, leading researchers to test other options such as arsenic and iodine. A breakthrough arrived with Dr. Upendranath Brahmachari's discovery of urea stibamine, a landmark discovery by a native doctor under the British state, was eventually adopted on a wide scale, advancing kala-azar treatment efforts across Eastern India.

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Review Article

Addressing research questions in visceral leishmaniasis and post-kala-azar dermal leishmaniasis: Potential of reusing data from the Infectious Diseases Data Observatory platform

Prabin Dahal[1,2], Sauman Singh-Phulgenda[1,2,3], Dawit Getachew Assefa[1,2], Philippe J. Guerin[1,2]

[1]Infectious Diseases Data Observatory, Oxford, UK, [2]Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK, [3]Coalition for Equitable Research in Low-Resource Settings, Paris, France

Abstract

The Infectious Diseases Data Observatory (IDDO) data platform was launched in 2016 with the aim of harmonizing individual participant data (IPD) from clinical trials on infectious diseases, with a specific focus on neglected tropical diseases, including visceral leishmaniasis (VL). The VL data platform hosted by IDDO was developed in collaboration with researchers at the frontline of disease combat. This platform currently hosts IPD from more than 50 studies on VL and post-kala-azar dermal leishmaniasis (PKDL). The platform remains a controlled open-access repository to the scientific community and is currently facilitating collaborative IPD meta-analyses addressing research questions regarding drug safety and efficacy. Further open-access resources maintained by IDDO include clinical case report forms (CRFs) for VL, and VL-human immunodeficiency trials, a comprehensive inventory of all published VL clinical trials (1980–to date) and PKDL trials (1973—to date), and these are publicly available as downloadable resources to the research community. These open-access resources developed in collaboration with the scientific community can help in identifying research gaps, and facilitate the generation of new evidence, and the CRFs can help to harmonize data collection for future trials. In this study, we highlight some of the ongoing and past research activities undertaken using these open-access resources (such as predictors of relapse and the development of a clinical scoring system for detecting relapse) and how these can support generating evidence to complement the ongoing activities in the Indian subcontinent and East Africa to reach the elimination targets.

Keywords: Kala-azar, leishmaniasis, PKDL

Address for correspondence: Dr. Prabin Dahal, Infectious Diseases Data Observatory, Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, Big Data Institute Building, University of Oxford, Oxford OX3 7LF, UK.
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BACKGROUND

"*The fell disease has mocked every human effort, and absorbed in its powerful grasp, day by day and inch by inch, every blessed spot which once used to be prized for its salubrity*"

UN Brahmachari, 1917[1]

Contrary to the quoted statement, Dr. Upendranath Brahmachari[1] would undoubtedly have made a much happier remark seeing the current progress made in visceral leishmaniasis (VL) control and elimination in the Indian subcontinent (ISC), 100 years later. Yet, this

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substantial decline in VL burden in the region is recent and was observed in the last two decades, providing renewed enthusiasm to the affected communities. Kala-Azar Elimination Program (KAEP),[2] that was launched in a cross-border collaboration between Bangladesh, India, and Nepal in 2005 played an instrumental role in affected countries to achieve elimination or are close to achieving the targets.[3] The success of KAEP and the lessons learned from the program have inspired a similar initiative across the Arabian Sea in East Africa.[4]

As global efforts are currently underway to preserve the strides made in the past decades in VL control, the research community needs to be cautiously optimistic as new threats are emerging as last-mile challenges, as observed for all disease-elimination projects.[5,6] Notable concerns include lack of a robust evidence for detecting the risk factors for VL relapses and the case management of those patients, recent ocular concerns following miltefosine regimen in post-kala-azar dermal leishmaniasis (PKDL) patients in the ISC challenges in identifying and managing asymptomatic cases, and limited alternative chemotherapeutic armamentarium currently available to existing drugs.[7-10] Addressing these existing and emerging challenges requires a coordinated effort from all the stakeholders including funders, policymakers, researchers, and patients alike. Such effort can be bolstered by a timely generation of evidence on drug safety and efficacy in a patient population with diverse characteristics, such as undernutrition and human immunodeficiency virus (HIV) co-infection, which remains crucial for optimal case management and safeguarding the patients. Continuously mapping the inventory of past and upcoming trials, that is, the development and management of a living database of clinical trials can be a useful tool to facilitate this. Such a living database serves as a "common good" to the scientific and the patient community and can help guide future research.

Open-access resources maintained by the Infectious Diseases Data Observatory (IDDO) are an example of such "commons" available to the scientific and patient communities. IDDO is a data platform that was launched in 2016 to harmonize individual participant data (IPD) from clinical trials on infectious diseases, with a particular focus on neglected tropical diseases (NTDs).[11] Before the development of a specific disease data platform, IDDO follows a set of established procedures, one of which is to undertake a systematic mapping exercise to identify past and upcoming trials, thus creating a unique inventory of published clinical trials for the disease of interest.[12-18] These inventories are then made available as open-access resources. Once the mapping is complete and the feasibility of the development of the data platform is established, IDDO actively engages with the research community collaboratively to further realize the platform through data solicitation and subsequent harmonization, which facilitates further data reuse.

Although IDDO was formally launched in 2016, the group's previous engagement with the global malaria research community as WorldWide Antimalarial Resistance Network facilitated a seamless knowledge transfer on several key skills required for the data platforms on NTDs to succeed.[19] This included developing a robust governance structure around data privacy, harmonization of disparate datasets into a common format using Clinical Data Interchange Standards Consortium (CDISC)-compliant format, and setting up an equitable and ethical data-sharing process facilitated by an independent committee of scientists and members of the global research community. Furthermore, details regarding the underlying structure/governance framework have been discussed elsewhere.[19-21]

In this review, we describe the open-access resources maintained by IDDO on leishmaniasis [Table 1], and highlight some of the realized projects based on these resources and the future scientific potential of the VL data platform.

IDDO'S OPEN-ACCESS RESOURCES

The IDDO VL clinical trials library, 1980–to date (open access database)

A systematic review of the scientific literature was initially undertaken in 2016 to identify the existing VL clinical trials (PROPSERO: CRD42021284622).[14] The review identified 145 trials published (1980–2016, n = 26,986 patients) with the majority of the studies published after the turn of the millennium. This review has been periodically updated since the first undertaking. An open protocol exists,[22] and the database of clinical trials including information on drug regimens, geographical regions, and several study meta-data are made publicly available as a downloadable resource through IDDO's surveyor.[22,23] The VL clinical trials library currently has indexed 160 clinical trials and this has facilitated several meta-analyses on the safety and efficacy of antileishmanial drugs.[24,25]

The IDDO PKDL clinical trials library, 1973–to date (open access database)

As for VL, a systematic literature review was undertaken to identify randomized, and nonrandomized PKDL studies in 2023 (PROSPERO: CRD42021295848). The review identified a total of 56 unique studies describing 2,486

Table 1: IDDO's open-access resource

Resource	Description
The IDDO VL clinical trials library	An open-access library of published VL clinical, maintained periodically by the IDDO team. The library is based on a systematic search of the literature with an open and transparent search protocol.[22] PROSPERO registration: CRD42021284622 The library is available through the IDDO's surveyor webpage: URL: https://www.iddo.org/tool/vl-surveyor
The IDDO PKDL clinical trials library	An open-access library of published PKDL clinical trials, maintained periodically by the IDDO team. The library is based on a systematic search of the literature with an open and transparent search protocol.[17] As opposed to the VL clinical trials library, the PKDL trials library indexes not only trials but also case-series. PROSPERO registration: CRD42021295848 The library is available through the IDDO's surveyor webpage: URL: https://www.iddo.org/tool/vl-surveyor
The IDDO VL data platform	IPD repository of published (or unpublished) VL trials standardized by the IDDO team to a Clinical Data Interchange Standards Consortium (CDISC)-compliant common storage format. The database remains open to the scientific community who can request access through the IDDO's data access system which is controlled by a Data Access Committee. URL: https://www.iddo.org/vl/data-reuse/accessing-data
Development of case report forms for prospective data collection for VL and VL-HIV trials (including user guides)	Working together with the Drugs for Neglected Diseases *initiative* and the global VL community, case report forms (CRFs) have been developed for uncomplicated VL and VL-HIV co-infection. A PKDL extension has been incorporated into the uncomplicated VL CRF. The CRF allows the prospective collection of data for clinical trials data in a standard format according to global data standards, facilitating the sharing of individual patient data (IPD) to address knowledge gaps, advance research, and support subsequent drug development. Accompanying user guides have been developed to go with the standard CRFs. These are publicly available on IDDO's website. URL: https://www.iddo.org/vl/crf

IPD: individual participant data, IDDO: Infectious Diseases Data Observatory, PKDL: post-kala-azar dermal leishmaniasis, VL: visceral leishmaniasis, HIV: human immunodeficiency virus.
All the URLs accessed on October 27, 2024

patients.[17] The majority of the patients enrolled were from the ISC, including 1,512 (60.8%) from India, 662 (26.6%) from Bangladesh, and 47 (1.9%) from Nepal. A majority of the studies were nonrandomized (48 observational studies), with only 8 randomized trials suggesting a limited evidence base for supporting treatment recommendations. Overall, the level of heterogeneity in drug regimens and methodological variability across the studies precluded any synthesis of safety and efficacy outcomes, and suggested prospective harmonization of the definitions and protocols will be beneficial to the research community.

The IDDO VL data platform (a controlled open-access resource)

The IDDO VL data platform currently hosts standardized datasets for published and unpublished studies in VL and PKDL.[26] As of August 2024, the platform currently hosts IPD from 17,452 patients from 10 countries from a total of 50 data submissions on VL and 8 on PKDL, including data from clinical trials and observational settings. The datasets are currently available in a single CDISC-compliant format, thus promoting interoperability and reusability of data. While the VL and PKDL clinical trial libraries are publicly available as downloadable resources, access to IPD hosted by IDDO is controlled by strict data governance. This means the primary investigators who have submitted data to the platform retain the right to decide the way the data is re-used in the future.[27] The primary investigators can choose one of the two data governance models (i) data re-use authorization delegated to the Data Access Committee, which comprises a panel of global researchers independent of IDDO and consists of members of the global infectious diseases scientific community,[28] or (ii) the primary investigators keep the decision of data sharing rights with them, which means they will review each data request and decide on whether to permit the re-use of their data.[27] Therefore, by adopting the controlled open-access policy, IDDO champions the "responsible data sharing" ethos so that the primary investigators are engaged in collaborative research and can decide the data re-use model.

The VL case report forms (open access resource)

IDDO has collaborated with the Drugs for Neglected Diseases *initiative*, and the global VL community, pharmaceutical industry, drug regulators, and key national and global health partners to collaboratively develop case report forms (CRFs) for VL along with the user guides [Table 1]. These CRFs allow harmonization of prospective

data collection for future VL, and VL-HIV trials, and remain publicly available through IDDO's website.

SCIENTIFIC USE CASES OF IDDO'S OPEN-ACCESS RESOURCES

The primary goal of the VL and PKDL clinical trials library was to systematically map the landscape of clinical trials to assess the feasibility of the development of an IPD platform and this has been largely realized.[14,17] Despite this, IDDO has been continuously maintaining the VL and PKDL clinical trials libraries by periodically updating them and making them open-access. The VL and PKDL clinical trials library and the VL IPD platform serve as a unique resource to the leishmaniasis research community. Here, we highlight some of the key research activities that have been completed or that are currently underway using these open-access resources. A comprehensive list of all the past and current research projects based on these resources is presented in Table 2.

Research aimed at addressing VL drug safety and efficacy-related questions

The VL clinical trials library has facilitated meta-analyses aimed at estimating the expected distribution of safety and efficacy parameters.[24,25] This has included developing a comprehensive inventory of all reported severe adverse events (SAEs; including mortality) for each of the major drug regimens.[24] The safety review undertaken in 2021 synthesized the reported SAEs and estimated a mortality rate of 0.068 deaths per 1000 person-days at risk during the treatment phase (variation across regions and patient populations).[24]

Further utility of the VL clinical trials library was highlighted by a review undertaken on treatment relapses. Relapse is a key clinical outcome from both patients' perspectives and from a broader disease control perspective. Several studies have raised concerns regarding whether a 6-month follow-up duration remains optimal for capturing late relapses.[36-38] To address this question, the IDDO VL clinical trials library identified studies with a follow-up duration longer than 6-months and a meta-analysis was undertaken. The meta-analysis estimated that a quarter of observed relapses would have been missed with a 6-month follow-up period ($n = 21$ studies).[25] This finding also echoed the observations from South Sudan, where it was found that over 15% of all relapses occurred after one year (regional heterogeneity needs to be further considered when interpreting this finding).[39] Failure to appropriately capture and treat late relapses (occurring after 6 months) would provide a reservoir to sustain disease transmission,[40] and hence, this finding adds growing support towards implementing a longer follow-up for capturing late relapses (although caution needs to be applied in adjudicating if a late relapse is truly a relapse or a re-infection).

Other research studies based on the clinical trials library have included collating evidence on VL during pregnancy, identifying gaps regarding current blood transfusion practices, and summarizing the methodological aspects of trial design and conduct [Table 2]. The latter has included characterization of the patient's spectrum such as sex

Table 2: Past and current research projects using IDDO's open-access resource

Resource	Project	Disease	Status
VL and PKDL clinical trials library	Serious adverse events following treatment and expected mortality within the treatment period	VL	Completed[24]
	Relapse following treatment and proportion of relapses that would be missed with a 6-month follow-up compared to a 12-month follow-up duration	VL	Completed[25]
	Sex composition of patients enrolled in VL clinical trials	VL	Completed[29]
	VL in pregnancy and mapping the evidence base	VL	Completed[30]
	Practice of blood transfusion in VL trials and triggers adopted for initiating transfusion	VL	Completed[31]
	Methodological aspects of clinical trials such as inclusion/exclusion criteria adopted, definition of primary and secondary endpoints, and analytical aspects	VL	Completed[32]
	Distribution of Lost-to-follow-up in VL clinical trials	VL	Manuscript in preparation
	Reported distribution of history of VL among patients presenting with a PKDL	PKDL	Ongoing [See Figure 1]
IDDO IPD platform	IPD-MA is aimed at identifying risk factors for relapse and other treatment outcomes	VL	Ongoing; protocol published[33]
	IPD-MA aimed at the characterization of the evolution of hemoglobin and other hematological parameters	VL	Ongoing; protocol published[34]
	Development of a prognostic model for identifying patients at risk of treatment relapse	VL	Systematic review completed[35]

IPD-MA: individual participant data meta-analysis, IDDO: Infectious Diseases Data Observatory, PKDL: post-kala-azar dermal leishmaniasis, VL: visceral leishmaniasis

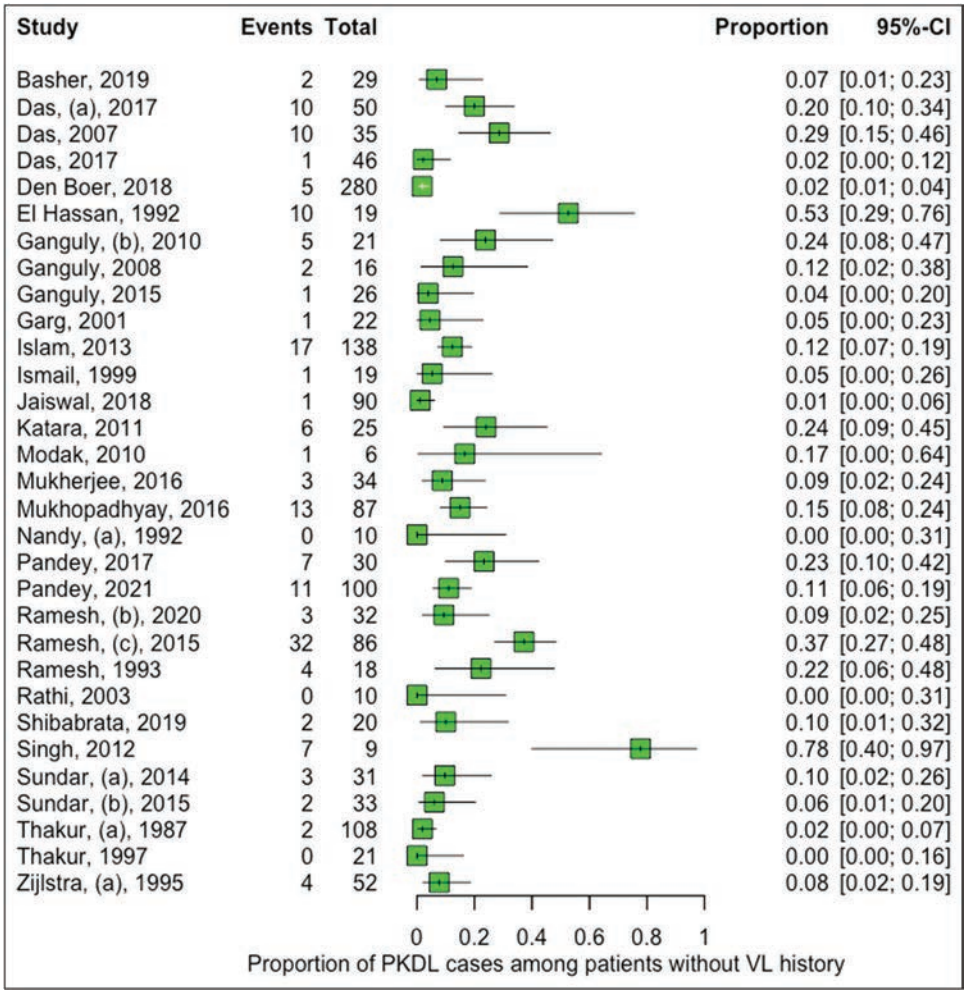

Figure 1: Proportion of PKDL patients without documented VL history in studies indexed in the IDDO PKDL clinical trials library (n = 30 studies with information reported).[17]

distribution,[29] and summarizing the inclusion/exclusion criteria adopted for patient enrollment along with several statistical considerations.[32]

The use of PKDL clinical trials library

The PKDL clinical trials library was developed in 2023, and hence, there has been limited scientific re-use thus far. The initial search identified 56 studies and a recent update identified an additional study. Overall, there are currently 57 studies indexed in the PKDL clinical trials library. Some key aspects of PKDL epidemiology based on the trial library are summarized next.

PKDL WITHOUT A HISTORY OF VL

Of the 57 studies, 27 (47.3%) studies explicitly reported the VL history of the patients and this information was unclear in the remaining 30 (52.7%). Overall, the 27 studies described 1,503 patients, of whom 1,331 (88.6%) had a documented history of VL, 166 (11.0%) had no documented history of VL, and this information was missing for 6 (0.5%) patients. The median proportion of PKDL patients without a VL history enrolled in a study was 9.7% [interquartile range (IQR): 4.2%–21.1%, range: 0%–78%; n = 27 studies] suggesting a substantial proportion of PKDL patients have no documented VL history [Figure 1]. This poses a particular challenge to disease elimination as PKDL can act as a reservoir of the disease.[40] Furthermore, research on this patient population remains a crucial aspect of leishmaniasis epidemiology and case management.

DURATION OF ILLNESS FROM APPEARANCE OF LESION TO HEALING

The average duration of PKDL from the appearance of the lesion to healing was reported in 14 studies. The distribution of average duration had a median time of 2.8 years with IQR between 2.5 and 3.9 years. Study-specific distribution is presented in Table 3. Additionally, the distribution of average interval from kala-azar treatment completion to

PKDL onset had a median of 5.2 years with IQR between 3.5 and 9.6 years (n = 18 studies). Study-specific distribution is presented in Table 4.

DISEASE SEVERITY

The definitions and the distribution of disease severity reported in the studies included are presented in Tables 5 and 6.

Table 3: Average duration of PKDL in studies indexed in the IDDO PKDL clinical trials library (n = 14 unique studies)

Author	Country	Mean disease duration (/years)
Mukherjee-2019[41]	India	4.4
Ghosh-2015[42]	India	8.4
Ghosh-2015 (a)[42]	India	6.8
Ghosh-2015 (b)[42]	India	4.1
Younis-2021[43]	Sudan	2.5
Moulik-2022[44]	India	2.5
Moulik-2022 (a)[44]	India	3.0
Musa-2005[45]	Sudan	2.3
Den Boer-2018[46]	Bangladesh	2.5
Mukhopadhyay-2016[47]	India	3.5
Musa-2002[48]	Sudan	0.8
Katara-2011[49]	India	5.3
El Hassan-1992[50]	Sudan	1.1
Moulik-2018[51]	India	2.9
Moulik-2018 (a)[51]	India	0.9
Garg-2001[52]	Nepal	2.6
Mukherjee-2016[53]	India	2.8
El-Hassan-1992 (a)[50]	Sudan	3.5

IDDO: Infectious Diseases Data Observatory, PKDL: post-kala-azar dermal leishmaniasis.

Table 4: Average duration of kala-azar treatment completion to PKDL onset in studies indexed in the IDDO PKDL clinical trials library (n = 18 unique studies)

Author	Country	Mean duration (/y)
Mukherjee-2019[41]	India	7.4
Ghosh-2015[42]	India	11.2
Ghosh-2015 (a)[42]	India	15.3
Ghosh-2015 (b)[42]	India	4.2
Ganguly-2015[54]	India	5.2
Modak-2010[55]	India	4.1
Thakur-1997[56]	India	3.3
Moulik-2021[44]	India	7.5
Moulik-2021 (a)[44]	India	5.5
Islam-2013[57]	Bangladesh	3.3
Ganguly-2010 (b)[58]	India	9.6
Den Boer-2018[46]	Bangladesh	5.8
Mukhopadhyay-2016[47]	India	3.5
Ganguly-2008[59]	India	3.6
Verma-2013[60]	India	10.8
Garg-2001[52]	Nepal	4.8
Mukherjee-2016[53]	India	2.5
Das-2017[61]	India	16.1
El-Hassan-1992 (a)[50]	Sudan	3.3
Ramesh-2020 (b)[62]	India	10.1
Singh-2012[63]	India	3.5

IDDO: Infectious Diseases Data Observatory, PKDL: post-kala-azar dermal leishmaniasis.

In Bangladesh, of the 778 patients from seven studies, three studies graded disease severity for 464 patients. Overall, 56 (7.2%) were grade I, 281 (36.1%) were grade II, 127 (16.2%) were grade III, and severity information was unclear in the remaining 314 patients [Tables 5 and 6]. In India, two studies reported disease severity for 76 PKDL patients, 48 (3.6%) were grade 0, 17 (1.3%) were grade I, and 11 (0.8%) were grade II PKDL with the classification status not reported in 1,257 (94.3%) patients. In two studies from Nepal, the disease severity of PKDL was ungraded.

In Sudan, of the 343 PKDL patients from 11 studies, only 4 studies reported lesion grades in a total of 214 patients of which 105 (30.6%) were classified as grade I severity, 71 patients (20.7%) were classified as grade II, and 38 patients (11.1%) were classified as grade III and classification was not reported in 129 (37.6%) patients [Tables 5 and 6].

LESIONS TYPE

From 1,503 patients, the total number of lesions reported was 1,728 (each patient can contribute multiple lesions). Of the 1,728 lesions reported, the most common were hypopigmented macules (43%, 742/1,728) and polymorphic (34%, 585/1,728). Other types of lesions, such as papular, macular, nodular, maculopapular, and papulonodular, were also reported and further details are presented in Table 6. In India, of the 1,004 lesions reported, 52.5% (527/1,004) were polymorphic and 33.8% (339/1,004) were macular. In Sudan, 38.1% (16/42) were papular and 33.3% (14/42) were maculopapular. In Bangladesh, of the 204 lesions reported, 61.3% (125/204) were macular and 15.5% (31/204) were maculopapular. In Nepal, 57.1% (32/56) were maculopapular and 42.9% (24/56) were macular.

Lesions primarily affected the face, trunk, and upper and lower limbs. However, other body parts such as the genitals, oral and nasal mucosa, tongue, soft palate, lips, dorsum of the palms, scrotum, torso, and soles were also affected [Table 6].

Ongoing collaborative IPD meta-analyses on VL

The VL data platform has provided a unique opportunity for the VL community to address questions regarding drug safety and efficacy. While many studies have previously aimed at identifying factors associated with treatment failures,[65-70] a robust assessment of such determinants is currently lacking as the number of relapses observed in any single trial is often small.[71] To address this research gap,

Table 5: PKDL disease severity definition adopted in studies included

Country	Severity	PKDL disease severity definition
Sudan	Grade I	Characterized by a dispersed macular, maculopapular, or nodular exanthema predominantly localized to the facial region around the oral cavity, with potential involvement of the upper thoracic area and proximal arms. Numerous small papules or occasionally larger ones may be observed.[33,35]
	Grade II	Characterized by a robust macular, maculopapular, or nodular exanthema covering a significant portion of the facial area, extending to the thorax, dorsal aspect, proximal arms, and lower extremities, with a gradual decrease in density distally, showing only isolated lesions on the forearms and legs.[33,35]
	Grade III	characterized by a pronounced macular, maculopapular, or nodular exanthema involving most of the body's surface, including the palmar and plantar regions. In grade three, crusting, ulceration, sloughing, scaling, and involvement of the mucosal surfaces of the lips (cheilitis) and the hard palate may occur.[33,35]
Bangladesh	Mild (1), Moderate (2), and Severe (3)	The highest severity score among scores for each type of lesion in each anatomical site for the individual.[57]
	Mild	Few lesions, not easy to spot.[46]
	Moderate	Easy to spot, plenty of normal skin.[46]
	Severe	Densely covered with lesions, hardly any normal skin to be seen.[46]

Post Kala-Azar Dermal Leishmaniasis. Further descriptions on PKDL grading can be found on the WHO Atlas[64]

Table 6: Lesion types and disease severity by countries

	Bangladesh	India	Nepal	Sudan
Lesion types	n=204 lesions	n=1,004 lesions	n=56 lesions	n=42 lesions
Polymorphic	32 (15.7%)	527 (52.5%)	0(0%)	1 (2.4%)
Papular	16 (7.8%)	62 (6.2%)	0(0%)	16 (38.1%)
Macular	125 (61.3%)	339 (33.8%)	24 (42.9%)	4 (9.5%)
Macularnodular	0(0%)	32 (3.2%)	0(0%)	0(0%)
Maculopapular	31 (15.5%)	21 (2.1%)	32 (57.1%)	14 (33.3%)
Papularnodular	0(0%)	23 (2.3%)	0(0%)	7 (16.7%)
Lesion location				
Total with information reported	409	164	11	23
Face	191(46.7%)	40 (24.4%)	4 (36.4%)	8 (34.8%)
Face, arm, and trunk	0(0%)	5 (3.0%)	0(0%)	12 (52.2%)
Face, arm, and leg	53 (12.9%)	15 (9.1%)	0(0%)	0(0%)
All body	46 (11.2%)	30 (18.3%)	3 (27.3%)	2 (8.7%)
Upper limb	80 (19.6%)	22 (13.4%)	0	0(0%)
Lower limbs	15 (3.6%)	24 (14.6%)	0	0(0%)
Genitalia	0(0%)	8 (4.9%)	4 (36.4%)	0(0%)
Trunk	0(0%)	18 (10.9%)	0(0%)	1(4.3%)
Lips	0(0%)	2 (1.2%)	0(0%)	0(0%)
Torso	24 (5.8%)	0(0%)	0(0%)	0(0%)
Disease severity graded?				
Yes	3	2	0	4
No	4	31	2	7
Patient with different severity				
Number of patients with disease severity information reported	778	1328	57	343
Grade 0	Not reported	48 (3.6%)	Not reported	Not reported
Grade I	56 (7.2%)	17 (1.3%)	Not reported	105 (30.6%)
Grade II	281 (36.1%)	11 (0.8%)	Not reported	71 (20.7%)
Grade III	127 (16.2%)	Not reported	Not reported	38 (11.1%)
Unclear	314 (40.4.1%)	1,252 (94.3%)	57 (100%)	129 (37.6%)

Column percentages are based on the denominator with the total reported information available.

the IDDO VL data platform is currently facilitating an IPD meta-analysis aimed at delineating risk factors for different therapeutic outcomes.[33] Another IPD-MA currently being undertaken has focused on the hematological aspect of the disease.[34] The critical mass of data hosted at the platform permits further opportunities for methodological development, such as the construction of a clinical algorithm that can be used to identify patients at a high risk of relapse or subsequent mortality. Such endeavour can lead to the development of an easy bedside tool to be deployed in field settings. Efforts are currently underway to facilitate the development of such a model.[35]

DISCUSSION

"*We still further look forward to the day when preventive medicine will eradicate this disease from India*"

UN Brahmachari, 1917[1]

The VL data platform now forms a critical mass of the data that has been generated over the past 20 years and,

essentially, the data that has been preserved in an electronic format to date. This platform is currently facilitating IPD meta-analyses on drug safety and efficacy.[33-35] This was possible due to the extensive collaborative effort between researchers across ISC, East Africa, Brazil, and the Mediterranean region. The evolution of the IDDO VL data platform, however, has spanned nearly half a decade, and during its development, there have been several challenges. In particular, there is a substantial logistical and time cost required to harmonize the data from diverse clinical trials to a common format and actively maintain this repository. Collaborative engagement of the research community towards supporting this common platform has been the hallmark despite the challenges incurred. Another key lesson has been that we weren't able to retrieve data from studies conducted before 2000. This serves as a reminder to the research community that the IDDO VL data platform can play an important role as a neutral venue for archiving and storing research data and prevent further knowledge loss. This is particularly relevant as there is currently only one new drug class in the phase II clinical trial for VL.[72] Furthermore, opportunities for the platform include the investigation of factors affecting treatment outcomes in PKDL or support of methodological innovation, such as using the platform to generate a historical cohort to facilitate new drug development.[73-75] The development of a new drug class is paramount as history has taught us that VL has re-emerged from near eradication with the disease exhibiting cyclical patterns of control and rebound in Bihar (India), often with a lengthy inter-epidemic period.[76]

PKDL poses significant challenges in Sudan, Bangladesh, India, and Nepal, mainly as a sequela of VL.[77-81] PKDL affects around 50% of VL patients in Sudan, often within 6 months of treatment,[80] while in India, 10%–20% develop it within 1–5 years. The disease is linked to family history and gender.[79,82] In particular, across the studies included in the IDDO PKDL trials library approximately 10% of the patients with PKDL presented without a documented VL history. Published literature has also highlighted a limited awareness of PKDL.[78,83] Taken together, this suggests that PKDL remains a notable challenge to disease control and elimination. The IDDO open-access resources, such as the VL and PKDL clinical trials libraries and the VL data platform, therefore, can be useful to the VL community to address some of the existing research questions, facilitate new drug development, and persevere the shelf-life of existing drugs through drug-specific IPD meta-analyses. This is particularly relevant as the limited funds available toward leishmaniasis control demand a judicious allocation and optimization of existing resources. The observed heterogeneity in key aspects of PKDL trials such as lesion types and disease severity grading also suggests a need for a comprehensive way to capture such data in future studies. For example, the VL CRF forms [Table 1] can be further extended to incorporate elements specific to PKDL trials so that data collection of future chemotherapeutic trials can be harmonized.

Dr. Brahmachari, who made several remarkable contributions to advancing VL chemotherapeutics and remained at the forefront of the battle against VL, would have undoubtedly maintained a hopeful outlook to the future as the disease is on the verge of elimination in the ISC. However, his bigger dream of "eradicating" VL remains a major challenge. The open-access resources maintained by IDDO can serve as "commons" to the scientific community towards realizing his dream of eradicating this "fell disease" (however infinitesimal contribution that might be).

Author contributions

PD and PJG conceived the study and wrote the first draft of the manuscript. DGA extracted and analyzed the data, and prepared all the tables and figures in the manuscript. All the authors were involved in reading and revising the initial draft and approving the final manuscript.

Availability of data and materials

This is a review article. All the data used in the review are available from the IDDO's surveyor website: https://www.iddo.org/tool/vl-surveyor.

Ethics approval and consent to participate

Not applicable.

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Conflicts of interest

There are no conflicts of interest.

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Review Article

Post kala-azar dermal leishmaniasis in East Africa, with a focus on Sudan: Review of three decades of experience and research

Eduard E. Zijlstra

Rotterdam Center for Tropical Medicine, Rotterdam, The Netherlands

Abstract

Post kala-azar dermal leishmaniasis (PKDL) is a well-known complication of visceral leishmaniasis (VL, kala-azar) in East Africa, with most cases reported from Sudan; the first description of PKDL dates back to 1921. Some three decades ago, increased interest (in Africa as well in other endemic regions) in PKDL was sparked through a letter published in the *Lancet* (1991) by the *Leishmania* research group of the University of Khartoum, describing PKDL in the absence of active VL. Shortly thereafter, a huge outbreak of VL was reported from the Upper Nile state and Gedaref state, which continues to date. Epidemiological studies reported PKDL incidences after VL in up to 50%–60% of VL patients, with age, poor nutrition, and inadequate drug treatment as the most important risk factors. However, the high PKDL rates were not uniformly found in all villages, where determinants such as tribal (genetic) background, socioeconomic circumstances, and exposure may differ. PKDL cases without preceding VL or with concomitant VL (para kala-azar dermal leishmaniasis) were described. Over the years, PKDL cases have been increasingly recognized with detailed clinical description, clinical staging, and combination of other post kala-azar manifestations, such as in the eye (conjunctivitis, blepharitis, and uveitis) and nasal mucosa. Diagnosis was initially done by clinically or microscopic techniques; later, the value of polymerase chain reaction (PCR) was demonstrated. Insight was gained in differential diagnosis, and, importantly, the evolving immune responses from VL (predominantly Th2) to cure (predominantly Th1) with PKDL as an intermediate condition (mixed Th2/Th1). The different immune response was also found to underlie the different most common forms: macular and papulo-nodular. Biomarkers were examined (clinical including imaging, parasitological, and serological and immunological parameters). A study about the natural history indicated that 85% of PKDL cases would self-heal, while other, more severe and persisting cases required treatment. Several studies were carried out to improve treatment outcomes of VL and PKDL with groundbreaking work in combined chemo and immunotherapy. While anthroponotic transmission of VL was assumed in PKDL patients as the reservoir, data on previous and recent entomological studies also provide evidence for zoonotic transmission. Important observations were made on sand fly biting behavior, strategies on the use of insecticide spraying, and the role of animals as zooprophylaxis and/or zoopotentation.

Keywords: Post kala-azar dermal leishmaniasis, epidemiology, diagnosis, immune responses, treatment, research priorities

Address for correspondence: Dr. Eduard E. Zijlstra, Rotterdam Center for Tropical Medicine, 3077 BB Rotterdam, The Netherlands.
E-mail: e.e.zijlstra@roctm.com
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BACKGROUND

Post kala-azar dermal leishmaniasis (PKDL) is a unique condition, which exists as an intermediate disease state between visceral leishmaniasis (VL), also called kala-azar, and clinical cure after treatment of VL[1,2]. While VL is a systemic disease characterized by fever, hepatosplenomegaly, pancytopenia, and wasting, in which *Leishmania* parasites may be detected in all organs, in PKDL, the patient is healthy and successfully recovered from VL, and the disease manifestations are normally restricted to the skin without any other symptoms or signs.[3] It is thought that PKDL occurs despite successful systemic treatment of VL as the result of inflammation surrounding parasites in the skin that have persisted from the VL episode.[4,5] This is the result of the evolving immune response toward *Leishmania* parasites. Indeed, the immune response in PKDL (mixed Th2/Th1) is intermediate between VL (absent, predominantly Th2) and definite clinical cure (predominantly Th1).[6-8] In most Sudanese patients, the immune response continues to evolve, resulting in self-healing of PKDL in 85% of cases within 13 months.[9]

PKDL is restricted to VL patients, caused by *Leishmania donovani,* and occurs in Africa, mainly Sudan and South Sudan, but also in Ethiopia, Kenya, Uganda, and Tanzania; and in Asia, mainly India, Bangladesh, and Nepal, with some different characteristics [Table 1].

In Asia, the correlation between PKDL with an evolving immune response after VL treatment is not clear; other conditions may be considered, such as intercurrent infections and their effect on the immune response to *Leishmania*, different *L. donovani* subspecies, and different genetic backgrounds.

In slit skin smears or biopsies, *Leishmania* parasites may be demonstrated, while these cannot be demonstrated in lymph node, bone marrow, or spleen aspirates as in VL. In both Africa and Asia, chronic PKDL patients played an important role in contributing to the outbreaks of VL as these patients may act as a reservoir of parasites; studies from Bangladesh have indeed demonstrated that sand flies become infected after feeding on 67% of PKDL patients, where nodular lesions more likely to result in positive xenodiagnosis (86%) vs macular lesions (35%).[10]

Search strategy

The electronic database PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) was searched for articles published till date (September 2024) using the terms "PKDL,," and "post kala azar dermal leishmaniasis." Relevant articles from the authors' personal files were identified, and from the articles, relevant information had been included.

Special forms

Para kala-azar dermal leishmaniasis occurs when PKDL already develops during VL treatment. In addition to the rash, the patient may develop fever, hepatosplenomegaly, and poor nutritional status. Parasites may be demonstrated in the lymph nodes, bone marrow, or spleen, as well as in the skin.[11]

PKDL without previous history of VL occurs in 10% of PKDL cases; this probably means that VL has not been recognized as an infection and may have been asymptomatic. Alternatively, the episodes of fever and splenomegaly may have been interpreted as, for example, a malaria episode.[12]

PKDL-like lesions in VL–HIV co-infection may occur preceding, during, or after VL infection or treatment; dermal lesions in HIV–VL co-infection may be a more appropriate term.[13]

Epidemiology

PKDL may have been detected in Sudan as early as 1921, although not recognized as such; Christopherson described a boy having VL treated with antimony tartrate, after which he developed "leukoderma" chiefly on the head and face and a "lichen planus" condition all over the body; both conditions disappeared spontaneously.[3] [Figure 1] Until 1990s, several authors reported on PKDL, and important steps were taken to confirm the diagnosis and to understand

Table 1: Most important characteristics of African and Asian PKDL

	Asia	Africa
Frequency after VL	10% (India) 20% (Bangladesh)	50-60% early reports; more recent 3-20% (Sudan)
Most important clinical presentation	Macular rash 90%	Papular rash 90%
Interval after VL (most common)	1->4 years (peak at 3 years)	0–13 months
Self-healing	Uncommon	Common, in 85%
Current treatment policy	All are treated	Only severe and chronic cases*
Possible role in transmission	Yes	Yes

*Currently under review; WHO recommendations expected early 2025

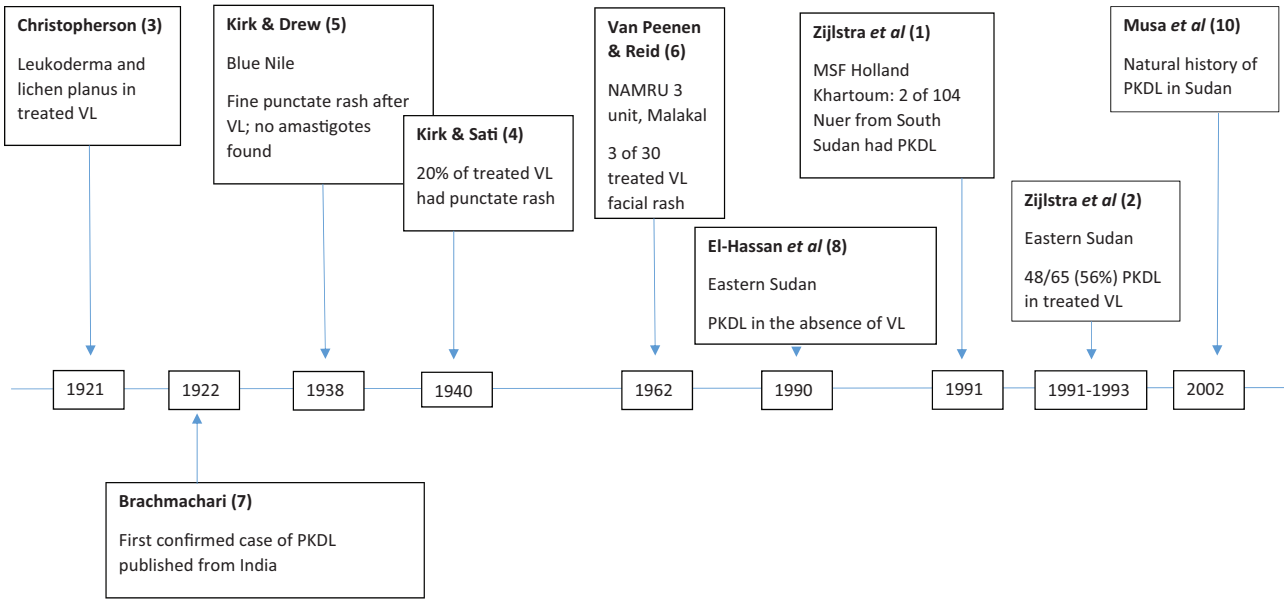

Figure 1: Timeline of landmark epidemiological studies reporting on the (presumed) diagnosis of post kala-azar dermal leishmaniasis (PKDL) in Sudan

the pathophysiology and the clinical and epidemiological implications. This occurred during outbreaks of VL but also in the interepidemic periods, of which the latter lasted until 1990. While touring the previously well-known endemic areas in Blue Nile and Gedaref states, the *Leishmania* Research Group of the University of Khartoum (LRG) did not find reports of VL cases from the local hospitals or from the inhabitants of the villages, of whom the older generation remembered VL very well. Screening of the villages also rendered no VL cases, but several PKDL patients were diagnosed. The LRG published a letter in the *Lancet*: "Post Kala-azar dermal Leishmaniasis in the absence of active VL," that sparked wide interest in this forgotten condition.[8]

Members of the Leishmania Research Group, Institute of Endemic Diseases, University of Khartoum, Khartoum, Sudan

Ahmed M. El-Hassan, Mohamed S. Ali, Dia A. Elnaiem, Isam A. Eltoum, Eltahir Fadl, Suha Gasim, Hashim W. Ghalib, Muntasser E. Ibrahim, Ahmed Ismael, Abdelgadir E. Elkadaru, Eltahir A. G. Khalil, Moawia M. Mukhtar, Ahmed M. Musa, Omran F. Osman, Maria Satti, Hiba S. M. Salih, Brima M. Younis, Eduard E. Zijlstra

All this changed dramatically from 1988 onward.

First, a huge outbreak of VL occurred among members of the Nuer tribe in western Upper Nile state in southern Sudan, concentrated around the town of Bentiu. This area was not known to be endemic for VL; the outbreak may have started already in 1984, but remained unnoticed because of the ongoing civil war. The disease was assumed to be introduced by Ethiopian soldiers, with whom the Nuer came into contact on the Sudan–Ethiopian border. As a result of this outbreak, given the lack of hospital facilities, a massive migration of Nuer tribe to Khartoum occurred, seeking treatment. This led to the establishment of the Kala-azar Hospital in El-Gerief, Khartoum, by MSF. In this study, the first cases of PKDL were diagnosed clinically and confirmed parasitologically.[1,14]

Between 1990 and 1991, 256 patients from the Misairiya tribe of Babanousa area in southern Kordofan were diagnosed with VL, most at Soba University hospital, Khartoum, and others locally. This nomadic tribe migrates every year with their cattle from Southern Kordofan southward toward Bentiu in the dry season.[15]

Second, a huge outbreak of VL occurred in Gedaref state, Eastern Sudan, from 1990; more than 50,000 treated patients were registered between 2002 and 2015.[16]

A longitudinal study was carried out by the LRG in the village of Um-Salala (population 1430) in Gedaref state, situated along the Rahad river. Over a study period of 2 years between 1991 and 1993, 48 (56%) of 85 VL patients diagnosed developed clinical PKDL; another 11 patients reported a transient rash, elicited from the histories

Table 2: Results of 6-monthly surveys between 1994 and 1996 in Mushrau Koka and Um-Salala villages: demographic details, number of visceral leishmaniasis (VL) cases, PKDL prevalence, nutritional parameters, spleen size, and malaria prevalence

Village	Mushrau Koka		Um-Salala	
Tribe	Hausa		Masaleet	
Established	1950		1969	
Place of birth	Mushrau Koka	Nigeria (Kano, Sakatu)	Um-Salala	Darfur, Sudan (El Geneina)
Number	754 (91%)	78 (9%)	543 (50%)	550 (50%)
Mean age	12.3 (11.2)	55.6 (15.1)	5.7 (4.7)	29.5 (15.5)
LST +ve	33%	88%	22%	88%
VL cases*	7	0	55	7
PKDL prevalence (mean of four 6-monthly surveys)	0–2.4/1000		6–13/1000	
Ratio clinical/subclinical**	1/2.5		1.6/1	
Z scores				
WAZ (weight for age *Z* score)	−1.27 (1.47)		−1.88 (1.2)****	
WHZ (weight for height *Z* score)	+0.09 (1.3)		−0.68 (1.12)****	
BMI (body mass index)	22.3 (9.0)		20.1 (2.8)****	
Spleen size (age <15 years)	1.37 (2.2)		0.49 (1.6)	
Mean spleen size in cm (SD)				
0 cm	64%		87%****	
1–4 cm	25%		8%****	
5–9 cm	11%		5%****	
10–14 cm	0.4%		0.3% ns	
Malaria prevalence***	72%		44%****	

Adapted from Khalil *et al.*[19]
*all cases occurred in those who were LST −ve previously
**Subclinical includes those converted in the direct agglutination test (DAT), LST, or both, without developing clinical symptoms
***In those eligible for testing: fever, illness, or splenomegaly; mean of four surveys with 6-month intervals
****$P < 0.001$
ns: not significant

Figure 2: Papular rash typically on the face

only. A surprising variety of clinical presentations was found, ranging from hardly visible and mild transient rashes to severe eruptions covering the whole body with systemic involvement and severe morbidity.[2, 12, 17] Similar PKDL rates were described in another study from eastern Sudan, where 16 (55%) of 29 VL patients developed PKDL.[18]

Surprisingly, epidemiological findings differed between two villages, only 35 km apart along the Rahad river. Compared to Um-Salala (Sudanese inhabitants of the Masalit tribe), in Mushrau Koka (MK), where people are of Nigerian origin (Hausa tribe), the incidence of VL cases and prevalence of PKDL were low, with a clinical-to-subclinical ratio of < 1. In contrast, malaria prevalence was higher with larger spleen size; the nutritional status was better. [Table 2]

Genetic factors, nutritional status, different ecology, exposure to sand flies, and other unidentified factors may play a role in explaining these differences.

Sex and age distribution

In Sudan, the sex distribution follows that of VL, in which factors leading to differences in exposure are important.[12] The mean age in VL and PKDL patients is around 6 years; PKDL is more common and more severe in very young children (0–4 years old).[2,12]

Description of the rash

The rash may be macular, maculopapular, micro-papular, nodular, plaque-like, or combinations of these. The lesions are not painful but may itch. Unlike in cutaneous leishmaniasis, ulceration is uncommon, which is in keeping with the much more moderate immune response in PKDL.

The maculo-papular rash is most common; in some cases, the papules are tiny, resembling the rash observed in measles. In other patients, they may increase in size and develop into nodules; these may become confluent over time and from plaques [Figure 2].

The macular type is characterized by hypopigmentation and well-demarcated edges [Figure 3].

As a rule, the rash starts on the face around the mouth and spreads to other parts of the face and upper limbs and trunk. In more severe cases, the legs are also affected. While in some cases, the rash remains limited to the face, others may present with a generalized rash.

A clinical grading system was developed to describe the severity and distribution at presentation and during treatment. [Table 3]

Figure 3: Macular rash affecting the perioral area and ear lobes

Images of PKDL can be found in the PKDL atlas: a manual for health workers, published by the WHO. (http://apps.who.int/iris/bitstream/10665/101164/1/9789241504102_eng.pdf)

Risk factors for development of PKDL

Risk factors for PKDL in Sudan can be grouped as follows:

1. Parasite – restricted to *L. donovani* infection causing VL in Africa and Asia. PKDL following *L. infantum* or *L. tropica* infection is uncommon, but dermal lesions may accompany VL–HIV co-infection.
2. Age – PKDL is more common and more severe in young children in age groups 3–6 years; this is likely the result of the still immature immune system.
3. Genetic factors – decreased function of the interferon–gamma receptor 1 gene (*IFNGR1*) was found to be linked to the development of PKDL in Sudan, while this was not demonstrated for VL. This was also detected in PKDL skin biopsies with a uniform low expression of IFN-γ and *IFNGR1*, which may explain the persistence of parasites.[21]
4. Drug treatment for VL – inadequate and irregular treatment of VL has been found associated with the risk of PKDL [Table 4].

Table 3: Clinical grading system to describe the distribution and density of the lesions

	Distribution	Density
Grade 1	Scattered maculopapular or nodular rash on the face, with or without lesions on the upper chest or arms	Scattered lesions
Grade 2	Dense maculopapular or nodular rash covering most of the face and extending to the chest, back, upper arms, and legs, with only scattered lesions on the forearms and legs	Moderate density with normal skin in between lesions
Grade 3	Dense maculopapular or nodular rash covering most of the body, including the hands and feet; the mucosa of the lips and palate may be involved, and crusting and scaling occur	No normal skin; confluent papules or nodules

From: Zijlstra EE. Biomarkers in post kala-azar dermal leishmaniasis. Front Cell Infect Microbiol. 2019 Jul 31;9:228[20]

Table 4: Cure rates for various VL treatment regimens, type of study, subsequent PKDL rate, and follow-up time

Treatment	Study	VL cure rate	PKDL		References
			Rate	Follow-up	
SSG irregular/inadequate dose	Case series	n.a./131	69%	12 m	[22]
SSG 20 mg/kg × 15 days	id	n.a./65	35%	12 m	[22]
SSG 10 mg/kg × 30 days	Comparative study	34/38 (89%)	nil	Mean 8.2 m	[14]
SSG 20 mg/kg × 30 days	id	23/29 (79%)	0.3%	Mean 7.2 m	[14]
SSG 20 mg/kg × 15 days	id	31/37 (84%)	0.3%	Mean 7.2 m	[14]
SSG 20 mg/kg + Paromomycin 15 mg/kg x 17 days	Retrospective cohort	63% (lost to FU 27%)	0.9%	6 m	[23]
SSG 20 mg/kg × 30 days	RCT	188/200 (94%)	13%	6 m	[24]
SSG 20 mg/kg + paromomycin 15 mg/kg × 17 days	RCT	328/359 (91%)	6%	6 m	[24]
Paromomycin 15 mg/kg × 21 days	RCT	167/198 (84%)	9%	6 m	[24]
Paromomycin 15 mg/kg × 28 days	RCT	17/21 (81%)	19%	6 m	[25]
Paromomycin 20 mg/kg × 21 days	RCT	16/21 (81%)	4%	6 m	[25]
Paromomycin 20 mg/kg × 14 days + miltefosine × 14 days	RCT	155/170 (91%)	3%	6 m	[26]
SSG 20 mg/kg × 17 days + paromomycin 15 mg/kg × 17 days	RCT	156/170 (92%)	14%	6 m	[26]
AmBisome 10 mg/kg single dose + SSG 20 mg/kg × 10 days	RCT	47/51 (87%)	4%	6 m	[27]
AmBisome 10 mg/kg single dose + Miltefosine 2.5 mg/kg × 10 days	RCT	40/45 (77%)	2%	6 m	[27]
Miltefosine 2.5 mg/kg × 10 days	RCT	38/51 (72%)	10%	6 m	[27]

Table 5: Clinical features associated with differential diagnosis

Differential diagnosis	Clinical features
Leprosy (all forms)	- Lack of sensation and thickened nerves are the hallmarks. - Lesions may have raised edges and central repigmentation - There may be satellite lesions. - There may be loss of eyebrows (madarosis)
Neurofibromatosis (papules and nodules)	- Among other symptoms, soft nodules on the skin that can increase substantially in size - Family history - Slow progression from childhood
Secondary syphilis (papules and nodules)	- Diffuse rash which frequently involves the palms of the hands and soles of the feet as well as the face. Not itchy - Sometimes macular lesions with a raised edge
Measles	- Very similar to micropapular PKDL; look for other symptoms of measles (fever, conjunctivitis, and Koplik's spots)
Acne	- Adolescent age - Greasy skin and comedones (papules with white head)
Lupus vulgaris (TB in the skin)	- Usually starts with painful lesions with a nodular appearance
Discoid lupus erythematosus	- Often presents as red, scaly patches - Often symmetrical; may occur in the butterfly area in the face - Develop into atrophic, hypopigmented scars
Miliaria rubra	- "Prickly heat"; tiny papules, mostly on the forehead, itchy - Typical observed in a child wrapped in too many layers of cloth
Vitiligo (macules)	- Depigmented, not hypopigmented - macules - Involvement of the central part of the back (not in PKDL)
Pityriasis versicolor (macules)	- Hypopigmented, dark or/and pink lesions - Mildly itchy - Becomes more obvious on exposure to sunlight - Affects the upper trunk more than the face, proximal portions of extremities, lower abdomen, and sometimes the neck - More common in hot and humid climates
Pityriasis alba (macules)	- Light-colored patches that seem to blend gradually into the normal appearing skin
Chronic arsenic poisoning (macules)	- Hypopigmented and hyperpigmented lesions, often with typical other complications of arsenicosis

Differential diagnosis

The differential diagnosis includes several conditions. [Table 5] The priority of diagnoses may differ depending on the region.

Diagnosis and biomarkers [for review, see Zijlstra[20]]

Clinical

In most endemic settings, diagnosis is clinical based on the previous history of VL, the interval after VL, and the typical rash. A maculo-papular rash is most common, starting on the face around the mouth, and spread to other parts of the face and head, to upper limbs and trunk, and eventually to the lower limbs, basically covering the whole body. While over time, the spreading may occur, in most patients, the rash remains restricted or shows limited extension. The progression of the macular rash is less predictable. In chronic cases, fibrosis of the skin may occur, leaving scars.

It is important to exclude concomitant VL as the treatment will be different; the patient will be treated for VL during which the PKDL lesions will also be treated simultaneously. (see below)

After the initial clinical diagnosis, a differential diagnosis should be considered with appropriate diagnostic tests, if available or appropriate. [Table 5]

Repeated clinical assessment may be carried out using grading [Table 3], use of a manikin on which the lesions are plotted, or various clinical and/or parasitological scores.[20]

Imaging is useful using two-dimensional photography, which requires stringent standardization to allow comparison. The images may be interpreted by independent reviewers.

Three-dimensional optical scanning is a novel tool; this user-friendly tool may be used in the field and requires a handheld scanner and a laptop. The PKDL lesions may be quantified in terms of surface, circumference, and diameter; in addition, the volume can be measured, including changes in size and color over time, with an accuracy of 0.5 mm.[28]

Parasitological

Microscopy. Ideally, the diagnosis of PKDL should be confirmed by demonstration of the parasite in a slit skin smear (SSS - preferred), or tissue biopsy, with limited sensitivity (50% and 77%, respectively).[29]

Molecular tools. Omran Osman pioneered the use of PCR in diagnosis of PKDL in Sudan; PCR yielded positive results in 83% of skin samples and 81% of lymph node

samples, while for microscopy testing, results for skin samples and lymph node samples were 30% and 17%, respectively. This not only showed the increased sensitivity of PCR in diagnosis of PKDL but also the (persisting) presence of parasites in lymph nodes without systemic symptoms and signs of VL reflecting the on-going healing immunological response.[30]

Quantitative PCR (qPCR) is increasingly becoming popular to quantify the parasite load before and after treatment; in a study from India, Moulik *et al.* showed that qPCR could distinguish between those who were successfully treated with miltefosine and those who failed after LAMB treatment, with this predicting relapse.[31]

Serological markers

Antibody-based tests such as the DAT and rK39 ELISA are not useful in diagnosis of PKDL as antibodies persist as the result of the preceding VL episode. In India, the rK39 strip test was tested directly on slit skin smear samples with good sensitivity but unknown specificity.[32] Using the same test on sweat samples showed high sensitivity and specificity (on healthy controls) of 97% and 95%, in VL and PKDL patients, respectively.[33]

Immunological markers

In landmark studies, Gasim *et al.* showed that 20 VL patients had detectable parasites in their (clinically normal) skin; those with high levels of interleukin-10 in keratinocytes and in PBMCs were at risk of developing PKDL 6–24 months after VL. A similar relationship was found between high CRP levels in blood at diagnosis of VL, suggesting that the development of PKDL could be predicted.[18,34]

The LST is an *in vivo* test to assess the cell-mediated immunity (CMI).

In Sudan, all VL patients tested demonstrated a negative LST.[17,22,35] In PKDL, the LST may be positive in 40–50% of patients; this is not different from treated VL patients who did not develop PKDL. However, in PKDL patients in whom parasites could be demonstrated in lymph node samples, the LST was positive in 11%, while in those with a negative lymph node aspirate, the LST was positive in 37%, showing a developing immune response with clearance of parasites.[22]

LST positivity rates differ according to PKDL grade; 39%, 25%, and 24% patients showed a positive LST in PKDL grades 1, 2, or 3, respectively, suggesting that the extent and the severity of the rash are determined by the cell-mediated immunity (CMI).

Natural history

The only publication on the natural history of PKDL in Sudan is from Musa *et al.* [9] [Table 6] A total of 134 patients with PKDL were described; the mean age (± SD) was 6.4 ± 3.0 years. The rash developed 0.5–13 months after VL, with a relatively rapid onset associated with severe disease. The patients were followed-up for 12 months. It was most severe in children aged ≤ 5 years, with no gender bias. Spontaneous healing occurred within 12 months in 84%, with a mean duration (± SD) of 9.7 ± 4.7 months. Those who self-healed were more likely to develop a positive LST and were less likely to have a positive DAT, suggesting a Th1 response. Those who persisted (16%) showed the opposite trend, that is, with lower LST and higher DAT positivity rates, suggesting a Th2 response. [Table 6]. The spontaneous healers tended to be older than those who had persistent lesions, with mean ± (S.D.) values of 6.5 ± 3.01 and 5.5 ± 2.8 years, respectively ($P < 0.05$).

Pathophysiology

Figure 4 shows the association between VL and PKDL. As a result of antileishmanial therapy, parasites are killed and an immune response develops against *Leishmania* parasites. Clinically, in the VL patient, hepatosplenomegaly disappears, with improvement in the nutritional status. Systemically, as cure ensues, immunity occurs, and a transition is noted from a predominantly Th2 response to a predominantly Th1 response.

In the skin, for reasons not well-understood, the conversion of the immune response may not be as profound, and inflammation occurs around the parasites persisting from VL, with a mixed Th2/ Th1 immune response. Factors that may play a role include: poor drug penetration in the skin,

Table 6: Prospective follow-up of 134 PKDL patients with the number of self-healers and persisters, and corresponding ratio of positive LST (leishmanin skin test) and DAT (direct agglutination test) compared with baseline

	Number	Duration	Mean age (years)	LST number pos/neg	DAT number pos/neg
At enrollment	134			87/47	107/27
After 12-m follow-up					
Self-healers (SH)	113 (84%)	9.7 ± 4.7	6.5 ± 3.0	101/12	50/63
Persisters (PS)	21 (16%)	16.6 ± 5.5	5.5 ± 2.8	7/14	19/2*

From: Musa *et al.*[9]

*comparison between LST and DAT positivity/ negativity rates – $P < 0.05$

genetic predisposition, and the influence of UV light that causes damage to dendritic cells, resulting in a Th2 response by inhibitory T cells [Figure 5].[36]

The immune response also plays a role in clinical manifestations. CMI is strongest in acute PKDL compared to chronic PKDL.[37] In macular lesions, CMI is strong with low parasite numbers and low antibody levels (only IgG1 is elevated), while in the polymorphic (papulo-nodular) form, the CMI is low, induced by TGF-β and IL-10, with higher levels of markers for regulatory T cells, and high antibody levels, including both IgG1 and IgG3 (markers for IL-10).[38] Immune complexes may play a role in VL and help in increasing the risk of subsequent PKDL.[39]

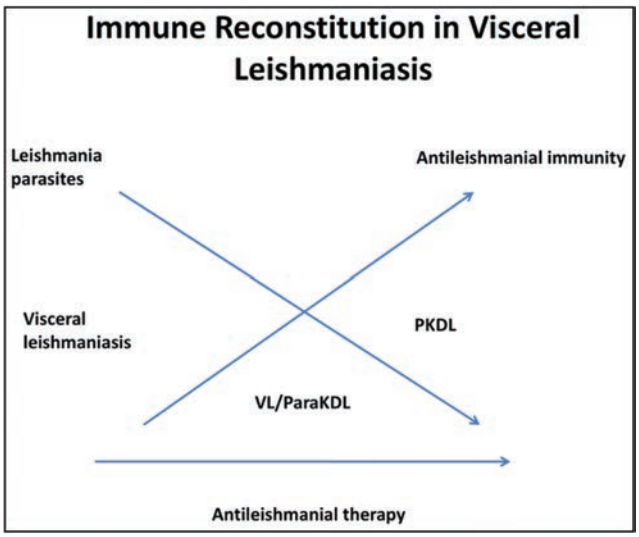

Figure 4: The inverse relationship between leishmania parasites and developing immunity during the treatment of visceral leishmaniasis (VL) and the corresponding clinical presentations. From: Zijlstra *et al.*[11]

Transmission

In the endemic area of Gedaref state, Eastern Sudan, *Phlebotomus orientalis* is the vector responsible for the transmission of *L. donovani,* causing VL. This vector thrives in environments characterized by black cotton soil and *Acacia seyal* and *Balanites aegyptiaca* trees. It is highly exophagic and exophilic and bites people in the household yard and in nearby peridomestic locations; this corresponds with the habit of sleeping outdoors and cattle herders spending the night in the field. The transmission is highly seasonal, and sandflies begin to appear after the rains in October and reach peak abundance between March and June before the heavy rains start.[40]

Infection rates of *L. donovani* in sandflies were found to be homogenous at different microhabitats in Eastern Sudan; the infection rate was 1.4%, sampled over 3 consecutive years with clear annual fluctuation between 0.0% in 2016 and 8.0% in 2018.[41] Blood meals in infected flies originated from humans (5 specimens), cattle (4 specimens) and

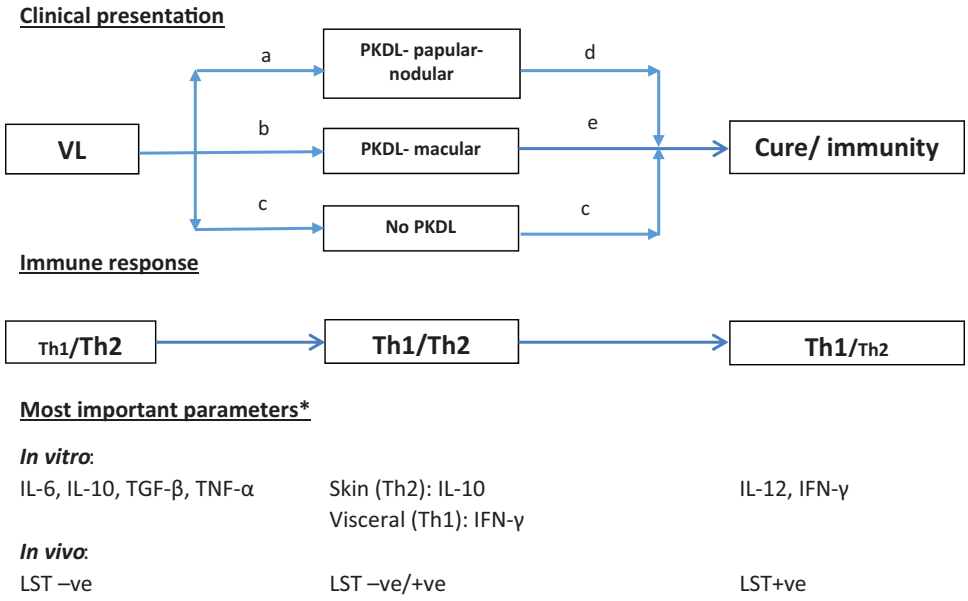

Figure 5: Schematic representation of clinical presentation, corresponding immune responses, and areas of missing information using the Th1/Th2 dichotomy. *Most important parameters that determine the immunologically relevant response. *a, b* the intervals differ in time between regions: 0-12 months in Africa; 0–3 years in Asia. There is limited information on the (timing of) developing or changing immune response in relation to the type of VL treatment (or in the case of absent VL history) and the clinical response (macular, papular, nodular PKDL; distribution and severity). *C,* the interval after successful treatment of VL and establishment of permanent immunity is unknown. *d, e,* there is no information on the (timing of) developing or changing immune response after PKDL treatment (or after self-healing), the clinical response (reduction or disappearance of lesions), and development of immunity. From: Zijlstra[37]

donkeys (2 specimens) suggesting the role of animals as a reservoir (zoopotentation) and/or zooprophylaxis. Earlier reports have demonstrated the presence of *L. donovani* in Egyptian mongooses that are found in the villages but also in an uninhabited site nearby Dinder National Park, suggesting a zoonotic transmission.[41,42] This issue is important as PKDL patients are assumed to be the most important (anthroponotic) reservoir for transmission of *L. donovani,* and a more comprehensive approach rather than treating PKDL patients to interrupt transmission may be necessary.(see below).

Treatment

While PKDL rates differ according to drug, dosing, and duration used, there are no data on VL treatment that may completely prevent PKDL.

Much less data on treatment of PKDL in Sudan exist. There is a dilemma on who should be treated as 85% of mild PKDL cases will self-heal, but who may contribute to transmission as a reservoir for *Leishmania* parasites.

It is important to define the clinical endpoint of treatment. [Figure 6] The clinical endpoint is flattening or decrease in the size of papules or nodules; for macular lesions, the decrease in size and re-pigmentation takes months in macular lesions and may be a poor marker for successful treatment outcomes. Immunological or parasitological endpoints are probably better, but these have not been established in studies. Like VL, PKDL was treated with sodium stibogluconate (SSG), but with a longer duration up to 60–120 days, until the rash had cleared. When the combination of SSG and paromomycin was introduced for VL for a duration of 15 days, PKDL patients were treated with the same regimen, but for 30 days with good results.[24,43]

This was confirmed in a large retrospective cohort study involving severe PKDL patients in South Sudan. In 343 patients who had received SSG monotherapy 20 mg/kg for 30 days intramuscularly (in primary healthcare units), the cure rate was 90%, while in 79 patients receiving a combination of SSG 20 mg/kg/day for a minimum of 30 days with paromomycin 15 mg/kg/day for 17 days, both intramuscularly, the cure rate was 97% (odds ratio 7.6; $P = 0.02$). The combination therapy had a shorter treatment duration (mean 34 days vs 42 days; $P = 0.005$) and lower defaulter rate (3% vs 9%; OR 0.3; $P = 0.03$).[44]

AmBisome treatment in PKDL was tested in 12 Sudanese patients with persistent PKDL lesions for > 6 months and no response on SSG therapy. Ten (83%) patients were clinically cured with AmBisome given intravenously at 2.5 mg/kg/day for 20 days, as evidenced by flattening of papular lesions or darkening (repigmentation) of macular lesions. There were no safety signals.

In a recent phase II trial conducted by the Drugs for Neglected diseases *initiative* (DND*i*), patients were allocated to two arms:

- Paromomycin (20 mg/kg, 14 days) daily intra-muscular plus oral miltefosine (allometric dose, 42 days) – PM/MF arm.
- LAmB (total dose of 20 mg/kg, administered in four injections in week 1) and oral miltefosine (allometric dose, 28 days) - LAmB/MF arm.
- Cure rates were 98% and 80% in the PM/MF and LAmB/MF arms, respectively.[45]

Immunochemotherapy has promising results; treatment with SSG and autoclaved *L. major* vaccine in PKDL patients who had failed previous treatment with stibogluconate

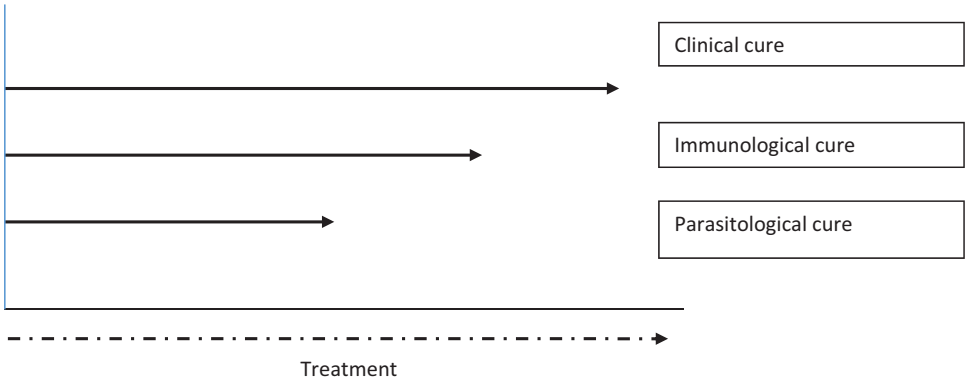

Figure 6: A simplified model showing the theoretical association between the occurrence of clinical cure, immunological cure (Th1 response), and parasitological cure after treatment in PKDL. The interval between these three outcomes and the duration of treatment is entirely hypothetical. From: Zijlstra *et al.*[50]

Table 7: Research priorities in PKDL and its role in transmission. [Adapted from reference (11)]

PKDL
•Priorities in diagnosis and biomarker
○Use of multiplex PCR in differential diagnosis
○Explore the use of artificial intelligence to recognize and (differential) diagnose
○Explore immunological parameters, e.g. a ratio of a pro- and anti-inflammatory cytokine
○Explore 3 -dimensional scanning as a biomarker
•Priorities in treatment
○Short, ambulatory, safe and effective treatment with aim of pushing the immune response towards a cure profile
○Explore use of biologicals
○Explore use of immune modulator or prophylactic/ therapeutic vaccine
•Priorities in prevention
○Optimal drug treatment for VL with lowest possible PKDL rate, including the understanding of immunological effects of antileishmanial drugs
○Explore pathophysiological trigger for late occurrence of PKDL in the ISC - intercurrent infection (helminths), loss of immunological memory (as in measles), other factors
○Prophylactic vaccine to be used in combination with VL treatment
Infectivity and transmission
•Establish uniform protocols (PCR, sand fly bites)
•Describe infectivity in the whole spectrum of VL and PKDL
•Describe changes in infectivity in PKDL early vs late presentation
•Determine infectivity in Africa in papular/nodular and macular PKDL, in early vs late development of PKDL, in acute vs chronic PKDL, according to age group
•Examine a possible animal reservoir in both East Africa and the ISC
•Develop model for interventions in Africa

resulted in 86% cure rate after 6 months, while this was 53% in those treated with SSG only.[46,47]

This concept was developed further in a new therapeutic vaccine ChAd63-KH, for VL, CL, or PKDL; it was shown to be safe and immunogenic in a phase 1 trial in the United Kingdom, and in a phase 2a trial in PKDL patients in Sudan.[48] A phase 2b study is underway.[49]

Dilemma

Treat all cases to interrupt transmission (as in ISC)

pro: important for control

contra: toxic drugs for non-ill patient, cost, long duration: current regimens not attractive; duration unclear

Treat only severe cases (as in Sudan)

pro: clinically indicated, thus side-effects more acceptable

contra: untreated cases persist for months/years: reservoir

The dilemma: while on one hand most PKDL patients in Sudan self-heal, on the other hand, the self-healing process may take 9 months, during which these patients may act as a reservoir for leishmania parasites, and this contributes to transmission. Indeed, the current outbreak in Eastern Sudan that started in 1990 continues to date.

New WHO guidelines on the treatment of VL and PKDL in East Africa are expected early 2025.

CONCLUSION

Research in PKDL in Sudan has made a tremendous impact on understanding the epidemiology, clinical presentations, pathophysiology, diagnosis, and treatment. Numerous scientific papers have been published. Other publications include a Supplement to the Transactions of the Royal Society of Tropical Medicine and Hygiene (leishmaniasis in Sudan. Vol 95, supplement 1, April 2001.[12] The Post Kala-azar Dermal Leishmaniasis (PKDL) Atlas, a manual for health workers, was published in 2012 (WHO/HTM/NTD/IDM/2012.4). Despite this, many questions remain unanswered.

The current research priorities in PKDL were recently described in the context of precision medicine and the control of VL caused by *L. donovani*.[51] [Table 7]

Declaration of patient consent

The author certifies that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflict of interests

There are no conflicts of interests.

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Review Article

Macular form of post-kala-azar dermal leishmaniasis: Clinical features, diagnosis, and significance in the Kala-Azar Elimination Programme

V. Ramesh, Nilay Kanti Das[1]

NDMC Charak Palika, Moti Bagh I, New Delhi, [1]Department of Dermatology, College of Medicine and Sagore Dutta Hospital, Kolkata, India

Abstract

Most of the hospital-based studies in post-kala-azar dermal leishmaniasis (PKDL) have been done in the polymorphic or mixed forms where the indurated lesions comprising papules and nodules played an important part in the clinical diagnosis rather than hypopigmented macules. Limited studies have shown that the monomorphic form of macular PKDL can be localized, generalized, or at times involve the entire body. Epidemiological work in kala-azar endemic areas has disclosed that in household surveys there are many with hypopigmented macules that were proven to be PKDL. The gender bias favoring males over females was also nonexistent, and the ratio of the clinical presentation of polymorphic and predominantly macular forms was equal. Both histopathology and slit-skin smears are not helpful in diagnosis; entomological studies have conclusively shown that the macules harbor the parasite though the load as seen in quantitative polymerase chain reaction (qPCR) studies is small. Thus, the macules do play a part in the spread of kala-azar. Treatment is the same as recommended for PKDL but since the hypopigmentation takes longer to repigment, better methods of test of cure like qPCR are required. Training modules for health workers doing epidemiological work must consider this presentation more seriously, increase awareness, and discuss other confounding dermatoses like pityriasis versicolor and vitiligo.

Keywords: Diagnostics, hypopigmentation, immune response, post-kala-azar dermal leishmaniasis, visceral leishmaniasis

Address for correspondence: Dr. V Ramesh, NDMC Charak Palika, Moti Bagh I, New Delhi 110 021, India.
E-mail: weramesh@gmail.com
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BACKGROUND

Post-kala-azar dermal leishmaniasis (PKDL), an uncommon sequel to treated kala-azar or visceral leishmaniasis (VL) is characterized by a variety of eruptions of which the hypopigmented macules and indurated lesions (papules and nodules) comprise the mainstay. The latter can be seen in combination with hypopigmented macules, but the stress on clinical diagnosis has often centered around the distribution of the indurated erythematous lesions such as papules, nodules, and plaques. This was attributed to the majority of PKDL cases presenting only when papulo-nodular lesions appear, which causes disfigurement and social embarrassment.[1] resulting in patients with only macules staying below the radar.[2,3] These nodules appear gradually on the face and extremities while on the trunk, arms, and thighs they remain as macules.[4]

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Brahmachari[5] first described the disease in 1922 and stated that in his experience "erythematous induration rather than hypopigmentation first appeared followed by nodulation." This may account for the less frequently seen monomorphic forms of nodular PKDL. Second, it had been observed that the lesions appear first as hypopigmented macules and some transform into papules and nodules over time accounting for a mixed or polymorphous presentation.[6,7] Consequently, the indurated lesions which are important for clinical diagnosis and laboratory investigations have been the subject of many studies but those patients presenting with predominantly macules or remain as only macules have received less attention. The hypopigmented macules, often said to be the earliest lesions, appear on any part of the body, the most common being the face, trunk, and extremities, and less common being the hands and feet. They are pin-pointed at first, arise in crops, increase to half an inch in diameter, and may coalesce to form larger patches. The whole body may be affected, and since the pigmentary loss is never complete as in leukoderma, it has been picturesquely described as a dark-skinned Indian becoming a fair Irani![4] However, even when the pigmentary loss is extensive, no organisms have been demonstrated in skin smears.[8] Rarely, hypopigmented macules have been described on the mucous membrane of the hard palate.[9] The normally hyperpigmented sites like the axillae, groins, and the belt area of the abdomen remain spared [Figure 1].[3,10]

Search strategy

The electronic database PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) was searched for articles published to date (September 2024) using the terms "PKDL," "post kala azar dermal leishmaniasis." Relevant articles from the authors' personal files were identified, and from the articles, relevant information was included.

Characteristics of macular PKDL

In a hospital-based study spanning two decades, it was noted that approximately a quarter of the patients presented with macular PKDL; when reviewed based on gender, the proportion of the macular form was found to be significantly higher in females than males.[11] Some of these patients had been part of an earlier report in which the varying presentations of monomorphic macular PKDL were studied. Based on the clinical features, they broadly fell into three groups, (i) localized, limited to the face [Figure 2], (ii) generalized, involving the face, trunk, and extremities in a bilaterally symmetrical fashion [Figure 3A–C], and (iii) extensive, sparing islands of normal skin [Figure 4A and b]. The generalized form was the commonest, whereas the involvement of the labia majora, glans penis, palms, and soles were the uncommon sites affected [12] This panoramic presentation of macular PKDL is distinctly uncommon in African PKDL where macular lesions have been rarely described in the face of children.[13]

The laboratory diagnosis of macular PKDL has always been difficult as slit-smears are often negative for

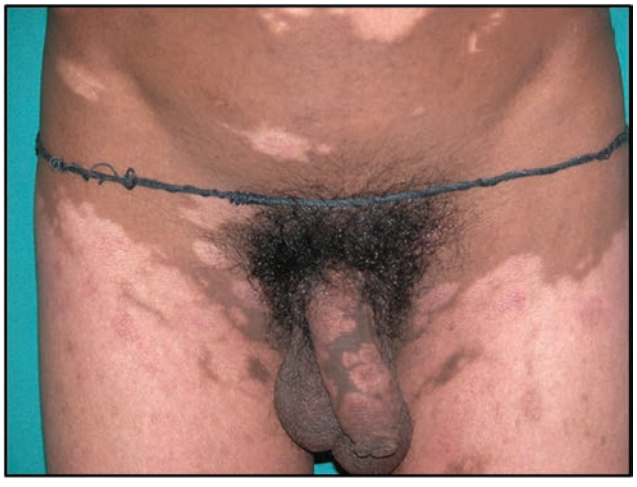

Figure 1: PKDL lesions classically sparing the middle of the back and belt area

Figure 2: Localized lesions of PKDL on the face

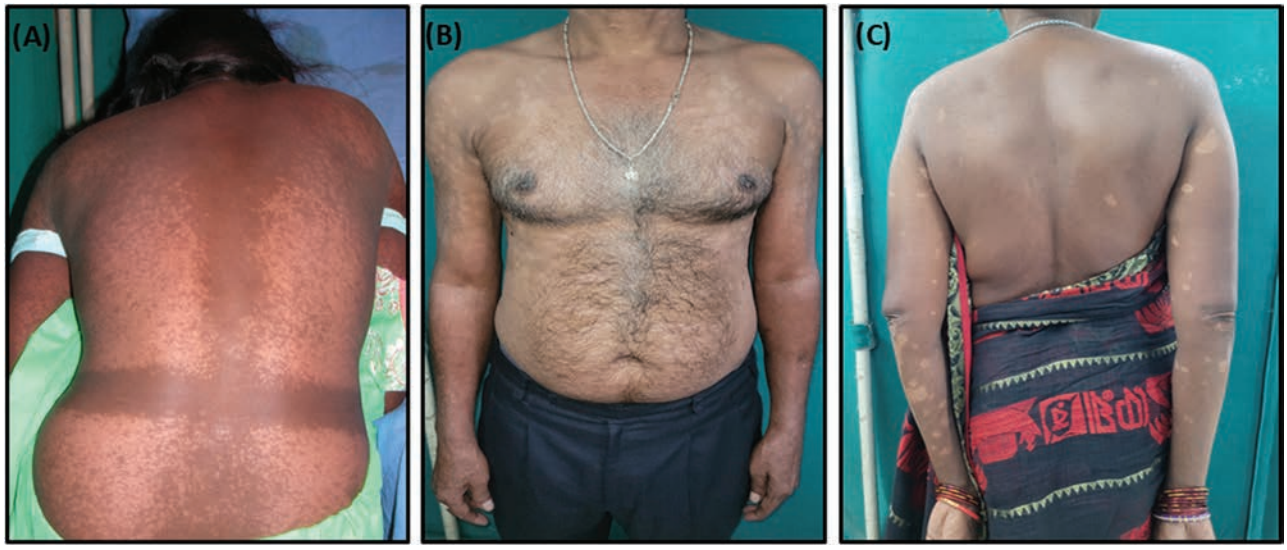

Figure 3: (A–C) Generalized involvement in PKDL in a bilaterally symmetrical fashion

leishmania donovani (LD) bodies.[10] From the earliest times, culture in the Novy–MacNeal–Nicolle medium has helped in isolating the parasite.[2] In another painstaking experiment, the authors made the sandflies feed on the macules and demonstrated the flagellate infection in the gut of the insect.[14-16] Histopathology revealed a mixed chronic inflammatory infiltrate of histiocytes, lymphocytes, and a few plasma cells, but LD bodies consistently defy recognition.[12] Immunohistochemical staining helped to identify LD bodies from some macules.[17] This challenge can only be resolved by utilizing molecular tools such as internal transcribed spacer region 1 polymerase chain reaction (PCR) and quantitative PCR (qPCR). Lastly, the advent of PCR improved the situation as 10 of 11 LD body-negative cases could be confirmed as PKDL.[18] Another challenging aspect is the lower parasite load in macular PKDL cases compared with the polymorphic form.[19] Although the diagnosis of PKDL mainly utilizes skin biopsies and slit-skin smears as parasites are found in the dermal lesions, blood has also been utilized for the diagnosis of PKDL, and using qPCR, the *Leishmania* specific kinetoplast deoxyribonucleic acid gene was identified with 77.50% sensitivity.[20]

Macular PKDL is particularly challenging to diagnose as clinical features are often indistinguishable from those of other hypopigmentary dermatoses prevalent in the same geographical area, including leprosy, pityriasis versicolor [Figure 5], and rarely even vitiligo [Figure 6]. The main clinical differential diagnoses of macular PKDL are macular leprosy and vitiligo. The macules in PKDL are small with irregular borders, seldom single, and form figurate patterns enclosing islands of normal skin, and pigment loss can be significant, whereas, in leprosy, the macules are bigger, may have sensory impairment and show moderate loss of pigmentation.[13,21] In other instances, histopathological findings of the composition of the infiltrate and its distribution would help in differentiating them.[12] Although nerve inflammation and peripheral nerve association are symptoms suggestive of leprosy, neural involvement has been reported in Sudanese PKDL[22] and in a case report from Bihar, wherein the case displayed lesional nerve infiltration and histologically mimicked tuberculoid leprosy.[23] Moreover, in a recent case report from West Bengal, a suspected tuberculoid leprosy case was confirmed to be macular PKDL by utilizing molecular tools.[24] On the other hand, vitiligo shows a complete loss of pigmentation or may show multiple shades of pigmentary loss in the same lesion; macules in PKDL are scattered on the trunk usually sparing the interscapular and midline areas until late in the disease.[25] Clinically, the other common similar conditions are pityriasis alba and pityriasis versicolor, lichen sclerosus, and rarely hypopigmented mycosis fungoides.

Detailed analysis of the comparison of the *in situ* immune profile of the two variants of PKDL-polymorphic and macular—in terms of their overall histopathology, especially concerning distribution and characterization of the cellular infiltrate at the lesional sites, has indicated several differences. In the macular form, the degree of cellular infiltration at the site of lesions is far lower than the polymorphic variant and importantly, a near complete absence of LD bodies.[26] The cellular infiltrate remained confined to the upper section of the dermis and the associated changes in the epidermis and dermis such as hyperkeratosis and focal papillomatosis were more often observed in the polymorphic variant. However, features like focal necrosis, a consistent feature in cutaneous

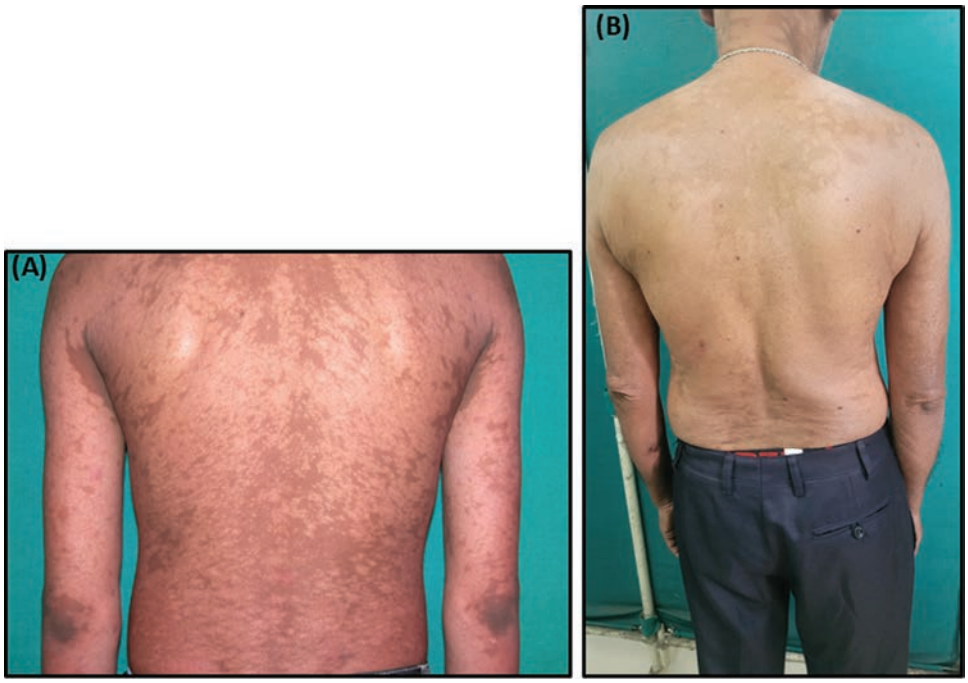

Figure 4: (A and B) Extensive involvement with sparing of island of normal skin

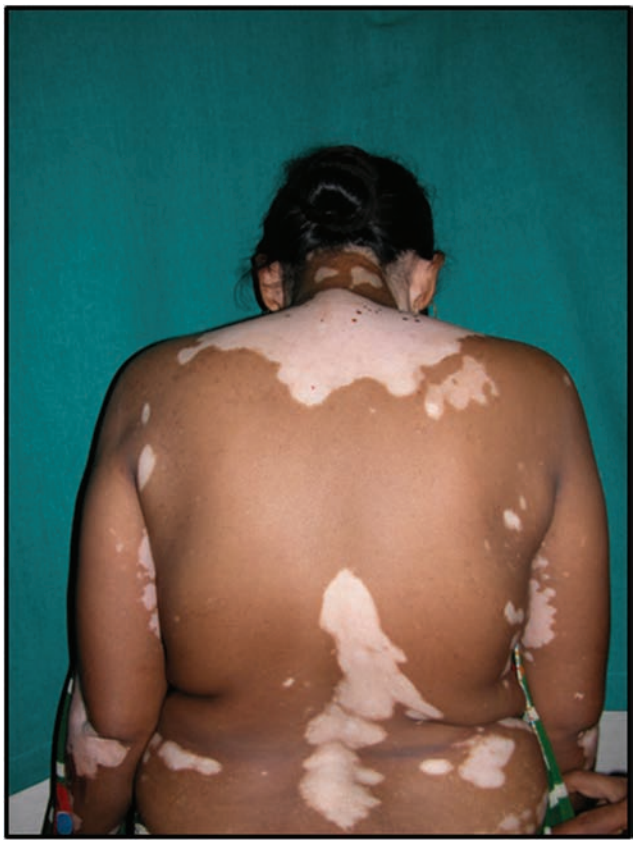

Figure 5: Vitiligo, a clinical mimicker of hypopigmented lesions of PKDL

leishmaniasis, or granuloma were absent in both forms of PKDL.[26] Furthermore, the generation of a relatively more balanced immune response and lower disease burden is evident in the macular form, as evident by a decreased infiltration of a cluster of differentiation (CD)68^+ macrophages, human leukocyte antigen—DR isotype, $CD8^+$ T-cells, and $CD20^+$ B-cells at lesional sites in comparison with its polymorphic counterparts.[26]

Mounting evidence has indicated that macules too play an important part in the spread of kala-azar.[16] Furthermore, epidemiological surveys as part of the National Kala-Azar Elimination Programme, have indicated that macular lesions predominate[27] compared with the mixed form. A similar study from Nepal showed that of the patients with hypopigmented macules referred to the hospital, <50% were found to be PKDL, and the remaining were pityriasis versicolor and vitiligo.[28] With the implementation of active surveillance (2015 onwards) in West Bengal, a huge number of macular PKDL cases were unearthed that translated the ratio of polymorphic and macular from 9:1[29,30] to 1:1.[31] Because of the cumulative scenario of PKDL, mimicking the active surveillance data that is, indicating a 50:50 distribution of macular: polymorphic forms, it is possibly a better representation of the lesional distribution of PKDL in India as compared to the previous reports of passive surveillance, that reported a>90% predominance of the polymorphic form.[32] Furthermore, a male preponderance in PKDL was reported during passive surveillance, whereas with active surveillance the scenario changed drastically as the ratio became 1:1.1, indicating the absence of any gender bias.[31, 32]

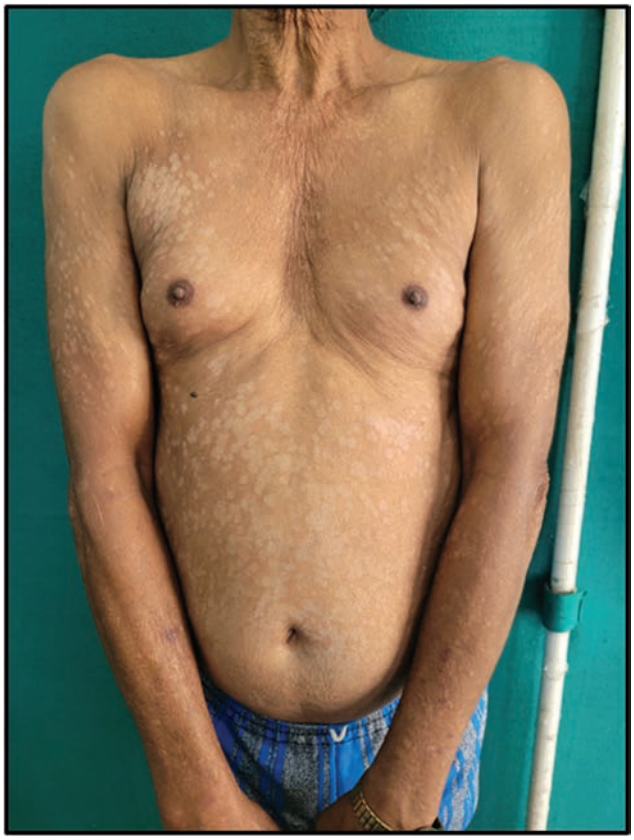

Figure 6: Pityriasis versicolor, a clinical mimicker of hypopigmented lesions of PKDL

The clinical readout for PKDL treatment efficacy is based on the resolution of lesions.[33] However, the primary limitation for macular PKDL is that despite completion of the 12-week miltefosine regimen by patients, hypopigmentation persists,[33] and repigmentation generally occurs at least 6–12 months later.[19] Consequently, molecular tools such as conventional PCR, qPCR, and sequencing can address this limitation, with quantification of parasite load being an unbiased parameter of treatment efficacy.[19, 34]

Although hypopigmentation is a consistent clinical feature in PKDL, contributory mechanisms responsible for this melanocyte loss remain unknown. The destruction of melanocytes is likely a multifactorial phenomenon, immune dysregulation being one of the key players as observed in other hypopigmentary dermatoses, like vitiligo, psoriasis, etc. This review focuses on the various immunological mechanisms. The prime suspects responsible for the loss of melanocytes at the lesional sites include (a) cytokines via creation of a pro-inflammatory milieu [e.g., interferon-gamma, interleukin (IL)-6, and tumor necrosis factor alpha], (b) chemokines by facilitating the homing of immune cells, and (c) $CD8^+$ T-cells possibly via their cytolytic function along with (d) keratinocytes that via decreased secretion of melanocyte growth factors, destruction of cell adhesion molecules, or via activation of the inflammasome signaling cascade can translate into IL-1β mediated melanocytic death. Unraveling the complex interplay between immune cells, cytokines, chemokines, and other immunological mediators, could provide new perspectives in understanding the immunopathogenesis of PKDL, and ultimately facilitate the identification of "host-directed" strategies for improved disease management.[35]

CONCLUSION

The National Kala-Azar Eradication Programme should take note of the macular presentation of PKDL in their training module for health workers. Since there are not many typical clinical features for diagnosis and the recognition relies heavily on experience, the differential diagnosis of these lesions must be well elucidated. At times, the performance of simple tests like slit-skin smears may help and could be done in instances where it is suspected. It may well prove to be the decisive battle in our efforts to eliminate kala-azar.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interests

There are no conflicts of interest.

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Review Article

Visceral leishmaniasis: Recent updates

Jaya Chakravarty, Amartya Seth, Shyam Sundar

Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Abstract

Visceral leishmaniasis (VL), a severe neglected tropical disease, presents a substantial global health burden, with an estimated 1 million new cases annually. Although cutaneous leishmaniasis (CL) is more prevalent, VL is the deadliest form, particularly in regions such as the Indian subcontinent, East Africa, and Brazil. The disease is caused by *Leishmania donovani* and transmitted through infected sandflies. Advances in VL management have significantly reduced the number of cases, particularly in India, Nepal, and Bangladesh. However, challenges persist due to human immunodeficiency virus-VL (HIV-VL) coinfection, which exacerbates disease severity and treatment resistance. Effective diagnostic techniques such as polymerase chain reaction and rk39 antigen tests are essential for timely identification of VL, though limitations persist in HIV-positive patients and asymptomatic carriers. Current treatment options, including liposomal amphotericin B and miltefosine, have shown high efficacy, with combination therapies offering promising results in addressing drug resistance and reducing the treatment duration. Post kala-azar dermal leishmaniasis (PKDL) poses a significant challenge to VL elimination, as it serves as a reservoir for ongoing transmission. Shorter, safer regimens are needed, particularly for endemic regions such as East Africa, where traditional treatments are less effective. Continued global collaboration is critical to achieve sustained progress in the elimination of VL and its complications, particularly for vulnerable populations affected by coinfections and drug resistance.

Keywords: HIV-VL coinfection, kala-azar elimination program, *Leishmania donovani*, liposomal amphotericin B, miltefosine, paromomycin, post kala-azar dermal leishmaniasis, visceral leishmaniasis

Address for correspondence: Prof. Shyam Sundar, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, Uttar Pradesh, India.
E-mail: drshyamsundar53@gmail.com
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INTRODUCTION

Leishmaniasis, a neglected tropical disease, accounts for nearly 1 million new cases globally every year. It is the second-most common cause of death due to parasite infection after malaria.[1] The clinical spectrum of the disease is diverse. It is caused by more than 20 protozoan parasites and is transmitted by the bite of an infected female sandfly of species *Phlebotomus* in the Old World and *Lutzomia* in the New World.[1]

Clinically, it can present as visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and muco-CL. While CL is the most common, VL is the most severe form of the disease, which can lead to mortality, if left untreated.

Every year, 30,000 new cases of VL are reported worldwide, most of which occur in Brazil, East Africa, and India.[2] The Indian subcontinent which was the main hub of the disease has made tremendous efforts toward the control of the disease. India which had reported 9241

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cases in 2014 has decreased the prevalence to 595 cases in 2023.[2] Nepal reported only 245 cases in in 2022, and Bangladesh has received WHO validation for eliminating VL in 2023.[3] HIV-VL infection has been reported from 45 countries, with high coinfection rates being reported from Brazil, Ethiopia, and the state of Bihar (India). Studies from India report that the prevalence varies between 2% and 5.6%.[4]

VL in the Indian subcontinent and Africa is caused by *Leishmania donovani*, and in the Mediterranean basin and central and south America, it is caused by *Leishmania infantum*. New species such as *Leishmania martiniquensis* and *Leishmania siamensis* have also been reported to cause VL as well as CL in Thailand and the French West Indies.[5-7]

Transmission of VL in South Asia and the Horn of Africa is anthroponotic, wherein patients with VL and PKDL are the main reservoirs. Transmission is zoonotic in the Mediterranean region, the Middle East, and Brazil, with the domestic dog as the most important reservoir. Rarely *in utero* transmission, spread through infected needles or transfusions, has been reported.

CLINICAL FEATURES

Visceral leishmaniasis

The incubation period is usually 2–6 months but can extend up to 3 years. Asymptomatic infections are common in endemic regions as the majority control the infection by mounting a successful immune response.[8] The disease affects all ages in the Indian subcontinent, while *L. infantum* predominantly affects the pediatric age group.

VL typically presents with fever, remarkable splenomegaly, weight loss, pancytopenia, and lymphadenopathy, with or without hepatomegaly. The incidence of lymphadenopathy is rare in the Indian subcontinent. Patients can also present with signs and symptoms of malnutrition, severe anemia leading to congestive heart failure, and thrombocytopenia leading to bleeding. Intercurrent infections such as measles, pneumonia, tuberculosis, and bacillary and amebic dysentery can occur with disease progression. Though the disease progression is typically sub-acute to chronic, at times, acute febrile illness can also occur along with rapidly progressive symptoms. In patients with prolonged illness, there may be blackish discoloration of distal extremities and skin and hence the name kala-azar (meaning black fever). Occasionally mild renal impairment can occur in adults and children affected by *L. infantum,* but some studies show that both the species causing VL can affect the kidney in the form of interstitial nephritis or glomerulonephritis on renal biopsy.[9] VL can also manifest in the form of secondary hemophagocytic lymphohistiocytosis, especially among children, and presents with fever with pancytopenia, organomegaly, and raised ferritin and fasting triglyceride levels.

Post kala-azar dermal leishmaniasis

PKDL is the dermal sequelae of VL and presents with maculo-papular nodules. The lesions are a combination of macules papules and/or nodules termed as polymorphic PKDL or hypopigmented patches termed as macular PKDL, predominantly appearing on the trunk, face, limbs, and on the back. It can have an associated regional lymphadenopathy especially in Africa. It occurs in East Africa in up to 60% of patients concurrently with VL or within 6 months, but most cases resolve spontaneously. In the Indian subcontinent, 2.5%–20% of patients with kala-azar develop PKDL within 6 months to 3 years after the cure of VL without spontaneous resolution.[10,11] It has also been reported in Brazil and HIV-coinfected VL cases caused by *L. infantum*.[12] PKDL infection serves as the reservoir for *leishmania* infection and thus poses a major challenge in elimination of the disease in the Indian subcontinent. Efforts taken by the kala-azar elimination program to actively search for PKDL cases has resulted in the decrease of cases from 1982 in 2017 to 324 in 2023 in India.

HIV-VL coinfection

As of 2021, *Leishmania*–HIV coinfection has been reported from 45 countries with high coinfection rates reported from Brazil, Ethiopia, and the state of Bihar (India). VL in People living with HIV (PLHIV) is usually more aggressive in terms of clinical features and leads to higher mortality due to the immune-compromised nature of the host. Also, the risk of developing infection is several times greater in PLHIV hosts.[13] VL is included in the WHO's clinical staging system for HIV as a stage 4, AIDS-defining condition. HIV-associated leishmaniasis has some special characteristics:[14]

1. Parasitic dissemination with involvement of almost every organ with reticulo-endothelial cells.
2. Atypical location of the parasite involving skin, gastro-intestinal (GI) mucosa, pleura, pericardium, and lymph nodes as a result of defective cell-mediated immunity.[15,16]
3. A chronic and relapsing course of illness.
4. Poor response to standard-of-care treatment due to decreased CD4 cell count.
5. rK39 and other antibody-based tests, although very sensitive in India, may be negative in up to 50% of the cases.

Clinical manifestations of these patients are usually same as found in immunocompetent hosts, but uncommon presentations can occur due to dissemination of the parasites to unusual sites (GI and oral mucosa, skin, pleura, pericardium, lymph nodes, and the respiratory tract).

DIAGNOSIS

The gold standard for diagnosis is demonstration of LD bodies in the splenic or bone marrow or lymph node aspirates. The sensitivity is highest for splenic aspirate (93%–99%), though the procedure carries bleeding risks which can be fatal and needs expertise followed by bone marrow (53%–86%) and lymph node aspiration (50%) samples. Cultures can also be done from these samples.

Molecular diagnosis of VL by polymerase chain reaction (PCR) in blood or bone marrow aspirates is very sensitive but is currently restricted to only referral hospitals and research centers. A portable loop-mediated isothermal amplification with a visual readout (LAMP) has been successfully developed, and it can be used in the most peripheral health facilities.[17]

Antibody-based tests have been used most commonly for the diagnosis of VL. Serological tests such as direct agglutination test (DAT) have demonstrated high pooled sensitivity (95%) and specificity of DAT (95%) but lack standardization.[18] The most commonly used test for diagnosis of VL, especially in the Indian subcontinent, is the rK39-based immunochromatographic test, which detects the presence of antibodies against the rK39 antigen. It exhibits very high sensitivity and specificity for VL in the Indian subcontinent, but lower sensitivity in East Africa, Latin America, and the Mediterranean region.[19] However, recent studies among pediatric VL in endemic areas of Europe have shown high sensitivity for this rapid test.[20] In the Indian subcontinent, the rK39 immunochromatographic test has been widely adopted as it is easy to perform, rapid, cheap, and yields reproducible results. Indirect fluorescent antibody test and TruQuick immunochromatographic test perform well in the Mediterranean region,[21] while the DAT is commonly used in East Africa. Antibody-based tests have some drawbacks as serum antibody levels remain detectable up to several years after cure, and therefore, they cannot be used to diagnose VL relapse. They may also be false-negative in HIV-positive patients. Thus, in cases of suspected relapse and PLHIV (people living with HIV), demonstration of LD bodies or PCR-based diagnostics has to be utilized. High prevalence of asymptomatic infections (up to 32% healthy individuals), living in endemic areas, may lead to positivity of serological tests. Thus, for the diagnosis of VL, antibody-based tests must always be used in combination with a standardized clinical case definition of VL. A "suspect" case of VL is a patient from an endemic area with a history of fever of > 2 weeks and enlarged spleen and liver not responding to antimalarials.

For diagnosis of PKDL, detection of parasites from lesions on stained smears, histopathology, and culture has low sensitivity and requires invasive procedures. Serological tests such as DAT, ELISA, and rK39 yield inconclusive findings for diagnosis of PKDL. PCR, real-time PCR, and isothermal assays, among others, have overcome these limitations being highly sensitive and specific nucleic acid–based tests. For an accurate and rapid diagnosis of PKDL, LAMP and recombinase polymerase amplification are emerging approaches.[21-25]

TREATMENT APPROACH

The armamentarium of antileishmanial drugs is small. Drugs that are active include pentavalent antimonial (Sb^V), amphotericin B (AmB), miltefosine, and paromomycin. Antimonial compounds were the drug of choice for treating VL worldwide for decades, but a high level of resistance emerged in the Indian subcontinent in the 1980s, while it remained active in other endemic areas. These drugs also induce life-threatening cardiotoxicity, pancreatitis, and hepatitis and are poorly tolerated by PLHIV, and that is why effort is on to move to other safer regimens.[1]

Conventional AmB, a polyene antibiotic, showed excellent efficacy against antimony-resistant VL in India. However, the regimen required 5–6 weeks of hospitalization. Adverse events such as infusion reaction, nephrotoxicity, hypokalemia, and myocarditis can occur, and thus close monitoring is required. Due to these reasons, the cost of therapy is high.

Many lipid formulations of AmB have been developed; however, liposomal AmB (L-AmB) is most commonly used in the treatment of leishmaniasis. L-AmB provides a targeted delivery to the reticulo-endothelial cells where *leishmania* reside and thrive. The adverse reactions are minimal and allow administration of a higher amount of the daily drug, decreasing the duration of therapy. The only drawback was the cost of the drug, which was prohibitive, limiting its application in large parts of the world. However, in 2007, a preferential pricing agreement between Gilead and the WHO led to the reduction of the price of L-AmB for endemic regions of medium- and low-income countries to $18 and later to $16 per 50 mg vial.

In 2010, a single dose of L-AmB at the dose of 10 mg/kg showed excellent efficacy in Indian VL as compared to the conventional AmB deoxycholate administered in 15 infusions of 1 mg/kg, given every other day.[24,26] These two events proved to be a game changer in the treatment of VL in the Indian subcontinent as the regimen became affordable, and a single dose ensured 100% compliance and decreased cost of hospitalization. After publication of this paper in 2010; in 2012, Gilead Sciences announced free donation of L-AmB (AmBisome) to countries (low/medium income) and is supplying the drug, extending the support till 2025. It was adopted as the treatment of choice for the Indian subcontinent by the WHO and the kala-azar elimination program. The total dose requirements of L-AmB vary from region to region. In the Mediterranean region and South America, L-AmB in a total dose of 18–21 mg/kg is the recommended regimen.[27] Recent studies in children with VL from the Mediterranean region have shown that short-course therapy with two 10 mg/kg doses of L-AmB is safe and effective.[28,29]

Miltefosine (MIL) is an alkyl phospholipid compound and is the only oral drug approved for VL. It was originally developed for the treatment of breast cancer and other solid tumors; however, it had dose-limiting GI toxicity. It was registered in India for VL treatment in 2002 following a Phase III trial in which 50–100 mg/day dose for 28 days resulted in a cure rate of 94%.[30] The advantages of oral administration, ease of use, and efficacy made it the drug of choice for the VL elimination program in India, Nepal, and Bangladesh in the beginning.[31] However, after a decade of use of the drug in the Indian subcontinent, its efficacy declined both in India and Nepal.[32-34] It is no longer used as monotherapy for VL in India but has shown excellent efficacy for PKDL and in combination therapy. Due to its teratogenic effect in animals and long half-life, it cannot be used in pregnancy, and contraceptives should be used in women of childbearing age for the duration of treatment and for a further 5 months.

Paromomycin (PM): It is an aminoglycoside–aminocyclitol antibiotic which was approved by the Indian government for the treatment of VL in 2006, at a dose of 15 mg/kg PM sulfate (11 mg base) for 21 days.[35] The efficacy of PM monotherapy was significantly lower in East Africa at the same dose and remained between 80% and 81% even after increasing the dose to 20 mg/kg or duration to 28 days[36,37] However the combination of PM with Sb^v for 17 days showed good results in Africa and is being used since 2002.[38-40]

Multidrug therapy

Multidrug therapy is advantageous over monotherapy and is one of the potential strategies for addressing the challenges associated with the treatment of leishmaniasis. This approach shortens the duration of the treatment, mutually protects each other against the development of drug resistance, reduces the cost of treatment, and shorter duration also results in greater availability of hospital beds. In India, L-AmB (one dose of 5 mg/kg) either combined with MIL (7 days) or PM (10 days) or a 10-day MIL plus PM treatment had an excellent cure rate of > 97%.[41] This could not be replicated in Africa as combination therapy with single dose L-AmB 10 mg/kg with 10 days sodium stibogluconate or 10 days miltefosine achieved an efficacy of only 87% and 77% at 210 days, respectively.[42]

A combination of sodium stibogluconate (SSG) 20 mg/kg/day with paromomycin (PM) 15 mg/kg/day for 17 days is recommended by the WHO as the treatment of choice in East Africa and Yemen.[27] This regimen is far from ideal due to the requirement of two daily injections, prolonged hospitalization, and toxicity of the regimen. Thus, there is an urgent need to decrease the duration of therapy and injections in Africa. Recently, in Africa, a combination therapy of MF plus PM for 14 days has shown similar efficacy to the standard of care (PM plus SSG regimen) in Africa. As this combination regimen is of shorter duration, requires one less injection daily, and induces lesser toxicity, it is likely to have better acceptability, especially among children.[43]

PKDL

The treatment drug of choice for PKDL in South Asia as per the National Vector Borne Disease Control Program Guidelines for Treatment of PKDL is miltefosine, with the dose being 50 mg daily for 12 weeks for adults weighing below 25 kg and 100 mg daily for 12 weeks for adults weighing above 25 kg. For children, the miltefosine dose is 2.5 mg/kg/day for 12 weeks. Both the treatment options have too long duration, are expensive, and can have toxicity, making it less acceptable as most patients of PKDL are asymptomatic. Moreover, in PKDL patients, serious ocular toxicities of miltefosine have been reported, such as ulcerative keratitis, leukocoria, blurred vision, ocular hyperemia, photophobia, eye pain, and rarely unilateral or bilateral blindness, mandating close monitoring.[44]

Efforts are ongoing to decrease the duration of treatment for PKDL. A recent phase II trial with 20 mg/kg L-AmB total dose, (five injections over 15 days) alone or combined with miltefosine for 3 weeks in India and Bangladesh

has shown better and satisfactory efficacy than found in previous PKDL clinical studies. Most lesions had resolved/improved at 12 and 24 months for patients receiving L-AmB (90%, 83%) and L-AmB/MF (85%, 88%) by qualitative assessment. There was no study drug–related serious adverse effects.[45]

In East Africa, PKDL is not routinely treated as most cases (85%) heal spontaneously within 1 year. Only patients with severe or disfiguring disease, those with persistent lesions (> 6 months) or concomitant anterior uveitis, and young children with oral lesions that interfere with feeding are treated. Sodium stibogluconate (20 mg/kg/day) for up to 2 months or a 20-day course of L-AmB at 2.5 mg/kg/day is used. In a recent phase 2 trial in Sudan involving pediatric patients, the combination therapy with MF (allometric dose, 42 days) + PM (20 mg/kg for 14 days) showed excellent efficacy with complete clinical response in 98% as compared to 80% in those receiving L-AmB (20 mg/kg) + MF for 28 days. This regimen potentially helps decrease therapy duration with one injection less.[43]

VL in PLHIV

A high dose of L-AmB 4 mg/kg on days 1–5, 10, 17, 24, 31, and 38 (40 mg/kg total dose) had been the treatment of choice for HIV–VL coinfection.[27] Recently, trials of combination of miltefosine for 14 days and L-AmB 30 mg on days 1, 3, 5, 7, 9, and 11 for HIV-VL showed excellent efficacy in the Indian subcontinent.[46] In East Africa, 28 days of miltefosine with 30 mg of L-AmB had better treatment outcomes than the conventional treatment.[47] Both the regimens have been recommended by the WHO as preferred options for HIV–VL coinfection.[48]

The WHO also recommends secondary prophylaxis for patients at high risk of relapse (e.g., patients not on anti-retroviral therapy (ART), with a low CD4 cell count (< 200 cells/mm^3), multiple previous VL episodes, failure to achieve clinical or parasitological cure during the first episode of VL, and no increase in CD4 cell count at follow-up). The regimen includes pentamidine isethionate at 4 mg/kg every 3–4 weeks in East Africa and conventional AmB at 1 mg/kg or L-AmB at 3–5 mg/kg per day every 3–4 weeks in South East Asia. Prophylaxis can be stopped if the CD4 cell count is maintained at > 350 cells/mm^3 or an HIV viral load is undetectable for at least 6 months and there is no evidence of VL relapse. ART should be started early to develop cell-mediated host immunity to prevent relapse rates and improve the parasitic clearance.

VL in pregnancy

VL in pregnancy can lead to fetal death and congenital infection. L-AmB is the treatment of choice for VL in pregnancy, and use of miltefosine is contraindicated.

CONCLUSION

Significant progress has been made in decreasing the number of VL cases, especially in the Indian subcontinent. This has been possible because of adoption of shorter and safer regimens for the treatment of VL like single dose of the L-AmB (10 mg/kg) regimen in this region; however, better management strategies are needed for PKDL. More needs to be done for VL in East Africa. Success of the combination regimen of MF plus PM for 14 days in Africa is a step toward shorter and more patient-friendly treatment for VL in Africa. The Americas have also shifted from toxic antimonials to a 7-day regimen of L-AmB for the first-line treatment of VL in 2022. Europe has found success with two 10 mg/kg doses of L-AmB for children with VL in the Mediterranean region. The success of combination therapy for HIV–VL coinfection has also been an important step toward management of this difficult-to-treat condition.

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Conflicts of interest

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Review Article

In vitro models in drug discovery and development for leishmaniasis: A perspective

Simon L. Croft, Katrien Van Bocxlaer[1]

Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, [1]Skin Research Centre, Hull York Medical School, York Biomedical Research Institute, Hull York Medical School, York, UK

Abstract

The intracellular *Leishmania* amastigote model (ILAM) has been instrumental in advancing drug discovery for leishmaniasis treatment over the past 40 years. This review explores the evolution and current applications of ILAMs in screening and drug development, focusing on its advantages for testing drug efficacy against intracellular amastigotes. Early models relied on macrophage cell lines, with notable progress made in the 2000s through molecular biology techniques such as bioluminescent and fluorescent transfection, enabling high-throughput screening (HTS) of large compound libraries. This method identified crucial drug leads, contributing to the extensive screening of over 4.5 million compounds by organizations such as the Drugs for Neglected Diseases initiative (DNDi). Recent advances in transfection technologies and CRISPR-Cas9 have further refined the potential of ILAM, allowing more precise genetic manipulation to assess parasite survival and drug resistance. Despite these advancements, challenges remain in maintaining *Leishmania* virulence in culture and addressing the variation in drug susceptibility across species. Furthermore, complex *in vitro* models and pharmacokinetic–pharmacodynamic (PK-PD) studies are being explored to optimize drug regimens and improve the predictive power of these models. While ILAMs have become a standard in drug discovery, their integration with more complex models and medicinal chemistry remains crucial for progressing toward an effective, short-course, oral treatment for all forms of leishmaniasis.

Keywords: Amastigote, drug development, *in vitro* model, leishmaniasis, PK-PD

Address for correspondence: Prof. Simon L. Croft, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK.
E-mail: simon.croft@lshtm.ac.uk
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INTRODUCTION

Over the past 40 years, the intracellular *Leishmania* amastigote model (ILAM) has helped transform drug and compound library screening. In this study, we review how the ILAM has been and can be used effectively to support drug discovery and development for leishmaniasis. The testing of compounds against intracellular amastigotes,[1] first used in dog sarcoma cells, progressed in the early 1980s when macrophages were used as host cells, starting with human transformed monocytes[2] and mouse peritoneal macrophages.[3] Since their use in 1980s, macrophage cell lines have become the established key host cell, with THP-1 cells first used in 1992[4] being predominant. Other cell lines have been used since the 1980s, but although infections were established with many *Leishmania* species, they often failed to show a reproducible infection and predictable division of *Leishmania* amastigotes. More recently,

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iPSC (induced pluripotent-derived stem cell)-derived macrophages, infected with *L. major,* demonstrate drug activities similar to those of other macrophage systems;[5] at present, the related cost limits wider use of this relevant model for screening.

Although there remains limited interest in the use of promastigotes (the easy-to-culture insect stage of Leishmania (for example[6]), the primary application of this model remains as a cytotoxicity indicator in bioassay guided fractionation of natural products.[7] The establishment of axenic amastigote cultures also showed some potential in drug/compound testing[8] and demonstrated similar activities of standard drugs to intracellular amastigotes. However, the use of macrophage cell lines, the introduction of transfected luminescent and fluorescent parasites, and high-quality imaging technology[8] has surpassed the results provided by all other models and expectations. This short review will focus on ILAMs and their use in screening and development of drugs for treating leishmaniasis. In the early days of ILAM use, we reviewed their potential and listed essential criteria that they must (i) provide active dividing populations of the mammalian stages of the parasite, (ii) provide a measure of drug activity that is readily quantified, and (iii) accurately reveal the activity of standard drugs at concentrations close to those achievable in serum or tissues (over a long time course if necessary). In addition, they should be able to examine additional features of interest, such as (iv) variation in drug susceptibility between strains and subspecies, drug resistance and (v) effects of immune or metabolic components.[9] The advancements in the past 40 years have redefined some of the criteria based on which ILAMs should be judged.

Screening and discovery

Although the original ILAMs offered the flexibility to measure dose responses, in comparing amastigotes/macrophage infection vs. % of macrophages infected, rate of kill, and dose–burden relationships, they had a major limitation of determination of infectious activity by using a manual light microscope and hence resulted in a low throughput. As such, they were not readily able to provide for high-throughput screening (HTS) needed for discovery based upon access to extensive commercial and pharmaceutical company compound libraries. The perspective of ILAM changed in the 2000s with the introduction of (i) molecular biological techniques allowing the use of stable transfected bioluminescent and fluorescent amastigotes.[10,11] Signal intensity can be registered using plate readers, but in more advanced settings, images are analyzed using algorithms. Even so, the software requires extensive training to ensure only amastigotes are taken into account as contamination with promastigotes can present a challenge – especially when evaluating the drug susceptibility of cutaneous leishmaniasis (CL) strains as these promastigotes can especially be difficult to remove and can contribute considerably to the signal (see below), (ii) advances in automated imaging techniques for 96 (and other)-well plates, and (iii) access to these larger compound libraries via organizations such as DND*i* (Drugs for Neglected Diseases *initiative*, Geneva) or directly from the pharmaceutical biotechnology companies. An early example of this approach[12] that used small commercial compound libraries confirmed its potential. In that following decade, large compound libraries from GSK[13] and Novartis[14] identified important leads from screens involving over 3 million compounds. The approach has changed the whole basis of anti-leishmanial drug discovery, with the efforts of DND*i* over 4.5 million compounds now screened. It is now the established mode.

Leishmania comprise over 20 species, responsible for a range of clinical manifestations, including cutaneous, mucocutaneous, and visceral leishmaniasis, which exhibit considerable variation in biochemistry, host–parasite interactions, and drug responses. Given these differences, it is crucial as a project moves into the development stage to include a defined diverse panel of species in research to represent the full spectrum of clinical outcomes. In their natural environments, *Leishmania* are amastigotes in the mammalian host, where they are constantly exposed to external stressors, such as temperature shifts, immune responses, and oxidative stress. Only the most adaptive parasites can survive these conditions, indicating that stress resistance plays a key role in their virulence and survival. However, when cultured in the laboratory, *Leishmania* tend to undergo changes, including impaired infectivity over time.[15] To maintain their virulence and ensure reliable experimental results, it is essential to regularly passage them through mice to preserve their pathogenic characteristics.

Two of the other original criteria of Croft[9] relevant to drug development have also been investigated within this new mode, namely: (i) the use of clinical isolates including those resistant to pentavalent antimonial, not just established laboratory strains,[16] and (ii) replicative rates of amastigotes using the bromodeoxyuridine analog, EdU, as a marker,[17] although the method of integrated rate of kill analysis, as established for *Plasmodium*, is still required. A refinement of this approach would establish both the division rate of amastigotes as well as whether the number of amastigotes in the macrophages also relates to intracellular killing or extracellular loss [Figure 1]. In addition, the application of tagged parasites also holds potential for novel assessments,

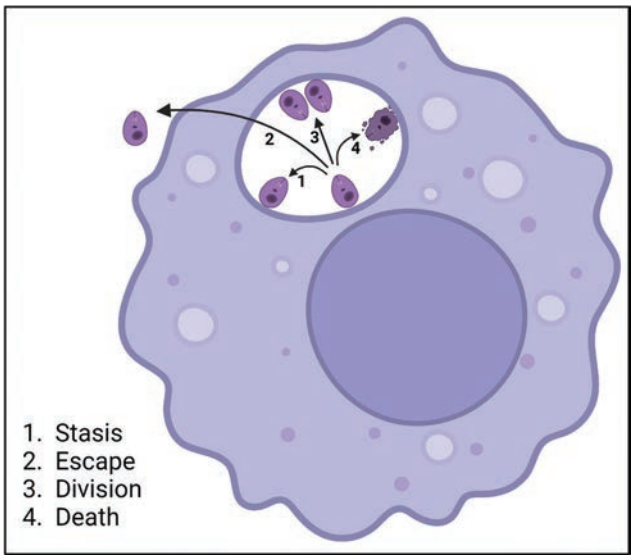

Figure 1: Possible fates of amastigotes within the intracellular Leishmania amastigote model (ILAM): (i) stasis, (ii) division (but rate not defined), (iii) death (but whether due to drug or macrophage killing factors not defined), and (iv) escape to extracellular environment (plasma, lymphatic fluid) [courtesy of R. Diaz and S. Croft, 17]

such as (iii) monitoring how the different parameters of the linear regression model vary over time and evaluating the drug effect on the parasite after a washout period[18] and (iv) the provision of a more dynamic understanding of drug–parasite interactions and resistance/persistence.[19,20] Furthermore, more recently, improved transfection methods and technologies, such as the use of CRISPR-Cas9, have facilitated precise genetic manipulation, enabling the modification or knock out of specific genes to assess their role in parasite survival, virulence, and drug resistance highlighting potential therapeutic targets.

Complex *in vitro* models

In a previous review on PK-PD, Croft[21] listed some of the factors that could impact upon infection of macrophages *in vitro* and *in vivo*.[21] Subsequent studies determined the degree to which some factors were of relevance. In ILAMS, comparison of drug activity levels in cultures maintained in a CO_2 incubator (O_2 level 18%) with those maintained under conditions used to culture *Plasmodium* (O_2 level 5%) in some compounds was impacted by oxygen tension (e.g., buparvaquone), whereas the standard antileishmanials were not (Croft unpublished). Further complexity was examined by O'Keeffe *et al.*[5,22] Infected macrophages in both VL and CL cases are subject to plasma or lymphatic fluid flow. In an ILAM *L. major* perfusion model (simulating *in vivo* lymphatic flow), there was a reduction of the infection rate of macrophages, the replication rate of the intracellular parasite, macrophage phagocytosis, and micropinocytosis, with greater reductions achieved under faster flow speeds. In this model, the activities of both amphotericin B and miltefosine were significantly reduced under perfusion flow compared to a static system, with an accompanying reduction in macrophage drug accumulation. 3D ILAM models were also included in these comparative studies.[5]

Although these studies showed the potential for further research using complex cellular assays and that they do not impact upon anti-leishmanial drug activities, but they are not sufficient to replace the current models used in HTS/HCS assays in drug discovery programs. However, their potential in drug development, along with organotropic models of the liver and skin, is yet to be fully explored.

In vitro models for PK-PD studies

An effort to de-risk and expedite the development of novel anti-leishmanial drugs for revealing an understanding of the relationship between the drug exposure (pharmacokinetics; PK) and the dose response to the agent (pharmacodynamics; PD) is key. For antibiotics, the relationship between PK and PD has been well-established, particularly in the context of time-dependent and concentration-dependent drugs. Extending this framework to anti-leishmanial drugs, Dorlo *et al.* associated the duration of plasma exposure to miltefosine above the *in vitro* EC_{90} (effective concentration) value with treatment success in VL patients.[23] However, the correlation between *in vitro* susceptibility and clinical efficacy for other drugs is still lacking. It is equally important to evaluate these markers in the context of CL as it is unclear if plasma concentrations are a suitable marker for parasite drug exposure in the skin.

Drawing further from research on antibacterial compounds, one- or two-compartment *in vitro* models could be used to mimic clinical findings and explore the PK–PD relationship between drugs and pathogens. For example, a one-compartment model is typically composed of a central reservoir, whereby the drug is eliminated by directing more drug-free medium into the central reservoir. This model could help simulate the effect of a drug half-life on a parasite population. We expand further to a two-compartment model, whereby drugs and nutrients are delivered and eliminated in the central compartment and exchanged via dialysis to the second peripheral compartment containing the pathogen. An example is the hollow fiber model that was used to simulate the contribution of both PK and PD parameters to resistance development in mixed bacterial populations upon exposure to a selection of antibiotics.[24] A combination of similar models and the ILAM could provide critical insights into how different drug regimens affect parasite clearance and resistance development [Figure 2].

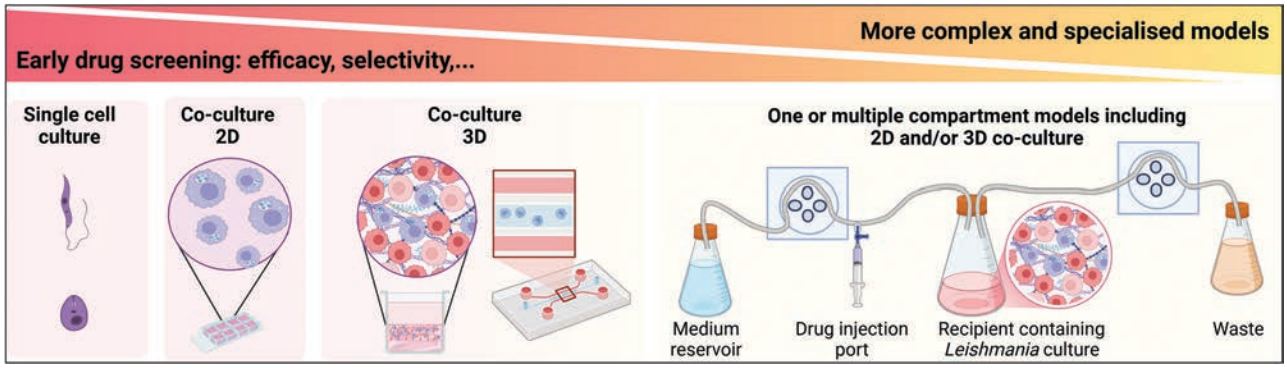

Figure 2: *In vitro* models based on the ILAM. From left to right: simplistic ILAM models adaptable to high-throughput screening used in the early stages of drug discovery to more complex 3D models (static or with medium flow) and fit-for-purpose one- and two-compartment models to evaluate intermittent drug exposure

Integration with medicinal chemistry and toxicity, permeability, protein binding, metabolism, and stability models

As compounds move through the screening stage and leads are identified and selected for lead optimization, it is essential to characterize as many medicinal chemistry properties as possible: cell membrane permeability, stability in serum at physiological temperatures, extent of protein binding, extent of hepatocyte metabolism, and toxicity profiles can all be characterized *in vitro*. This has been well-reviewed,[25,26] including the definition of the "Minimum information about a bioactive entity (MIABE)" required. This has been the normal procedure in pharmaceutical, biotech companies, and some academic centers over the past two decades. In relation to *Leishmania,* some elements have been established for a specific screening process, especially for CL.[27] However, access to these essential assays is often limited for most academic research works. Thus, the identification of centers or companies that can provide such is important. The novel technologies are applied in in the UK at Queen Mary University (https://www.cpm.qmul.ac.uk), where organ-on-on chip models at their *in vitro* predictive model center are available for this purpose; they also support the reduced use expensive animal models.

CONCLUSION

Although there has been considerable progress in drug R & D for leishmaniasis over the past two decades, the safe oral short-course drug that can treat all forms of leishmaniasis, or even VL alone, remains elusive. This is despite the efforts of DND*i* who have taken many leads into development (https://dndi.org/diseases/visceral-leishmaniasis/projects-achievements/), the participation of big pharma and biotech companies, the establishment of key academic centers with many skills, and a collaborative approach across these sectors. Hence, further discovery and screening are still required with the established HTS models. Within the development space, medicinal chemistry, pharmacokinetics and toxicity studies have been part of the research programs of DND*i* – pharma – biotech – key academic centers for most of this past two decades of progress. A key issue to support development is the improved understanding and integration of PK-PD models.[21,28,29] The role of *in vitro* studies, in particular the role of ILAMs, in determining relevant PD parameters needs to be taken further in relation to (i) rate of action, kill curve analysis of compounds, (ii) the actual intracellular and intravacuolar concentration to which the amastigote is exposed, (iii) potential intramacrophage metabolism of candidate compounds, and (iv) how all these factors are modified by activation of different types of macrophages by immune status. The *in vitro* model criteria for drug discovery and screening still stand, and those criteria for drug development remain under consideration.

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Nil.

Conflicts of interests

There are no conflicts of interests.

List of Abbreviations

CL: cutaneous leishmaniasis
EC: effective concentration
HTS: high-throughput screening
ILAM: intracellular Leishmania amastigote model
iPSC: induced pluripotent-derived stem cell
MIABE: minimum information about a bioactive entity
PD: pharmacodynamics
PK: pharmacokinetics
VL: visceral leishmaniasis

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Review Article

Mapping the immune landscape in South Asian post kala-azar dermal leishmaniasis

Madhurima Roy†, Ritika Sengupta†, Mitali Chatterjee

Department of Pharmacology, Institute of Post Graduate Medical Education and Research, Kolkata, West Bengal, India

Abstract

Leishmaniasis is a neglected tropical disease affecting the world's poorest populations in over 90 countries, of which visceral leishmaniasis (VL)/kala-azar is the most fatal. Post kala-azar dermal leishmaniasis (PKDL) is a chronic dermal sequela that occurs in patients who have undergone treatment for VL caused by *Leishmania donovani*. The geographical distribution of PKDL involves East Africa, where it presents as papular or nodular lesions, and South Asia, where it presents with widespread polymorphic and macular lesions. Patients with PKDL represent an important but largely neglected reservoir of infection that perpetuates anthroponotic *Leishmania* transmission and can jeopardize the VL elimination program in the Indian subcontinent. Therefore, although not life-threatening, it becomes imperative to diagnose and treat PKDL cases as a part of the elimination program, which, in turn, requires the availability of robust data regarding the immunopathology of dermal lesions. However, in the absence of an animal model, understanding the immunological basis of PKDL is critical for developing effective treatment regimens, but the information remains limited. A complex interplay between dendritic cells, neutrophils, macrophages, T cells, B cells, and associated cytokines plays a significant role in disease pathogenesis. Furthermore, recent findings have indicated differential immune responses between the two clinical forms of PKDL, which may impact on the disease pathogenicity and response to chemotherapy. Collectively, this review highlights the role of immune dysregulation in South Asian PKDL, which have allowed parasite persistence, leading to disease progression. Interventions via targeted immunomodulatory therapies aiming to restore effective immune responses could provide promising therapeutic strategies for management of PKDL.

Keywords: Adaptive immune cells, immune response, innate immune cells, leishmaniasis, post kala-azar dermal leishmaniasis, visceral leishmaniasis

Address for correspondence: Prof. Mitali Chatterjee, Department of Pharmacology, Institute of Post Graduate Medical Education and Research, 244B AJC Bose Road, Kolkata 700020, West Bengal, India.
E-mail: ilatimc@gmail.com
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BACKGROUND

Leishmaniasis is a vector-borne neglected tropical disease caused by intracellular protozoan parasites, of which 20 species of *Leishmania* can cause human disease and are transmitted by the bite of infected female phlebotomine sandflies. This disease occurs in three main clinical forms: (i) life-threatening visceral leishmaniasis (VL) or kala-azar with its dermal sequela, post kala-azar dermal leishmaniasis (PKDL), (ii) self-healing or chronic cutaneous leishmaniasis (CL), and (iii) mutilating mucosal or mucocutaneous

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†These authors are joint first authors.

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leishmaniasis.[1] The disease mostly affects population in low-income countries, with an estimated 7,00,000 to 1 million new cases occurring annually, and is associated with malnutrition, population displacement, poor housing, a weak immune system, and lack of financial resources.[2] In the South-East Asia Region, VL is endemic in Bangladesh, India, and Nepal, with sporadic cases also reported in Bhutan, Sri Lanka, and Thailand.[1]

Kala-azar/VL, the most fatal form of the disease, is characterized by prolonged fever, weight loss, weakness, anemia, and hepatosplenomegaly and, if untreated, results in mortality. Devastating epidemics of kala-azar have been recorded in the Indian subcontinent, namely, Bangladesh, India, and Nepal, since the early nineteenth century. However, over the past 15 years, endemic countries in the South-East Asian Region have made unprecedented strides toward kala-azar elimination.[3] In absence of vaccines to prevent VL infection, control of the disease relies on early diagnosis and appropriate treatment of patients to contain human reservoirs for the infection, along with vector control. In this regard, the emergence of PKDL as a dermal sequela to VL has raised concerns.[4] PKDL, which occurs in apparently cured VL patients, caused by *Leishmania donovani* (50%–60% cases in eastern Africa and 2.5%–20% cases in southern Asia, out of which approximately 15%–20% of the PKDL cases are reported from endemic areas without prior history of VL), is characterized by a cutaneous rash on exposed body parts, such as the face, ears, and hands, and can also affect other areas of the body during progression.[3,5,6] The role of PKDL patients as a reservoir for VL transmission is of utmost significance as a single case of PKDL has the potential to trigger a new outbreak of VL.[4,7,8] Therefore, continuous monitoring, early detection, and effective treatment of PKDL are crucial during the maintenance phase of the kala-azar elimination program (KAEP).

In PKDL, an important lacuna is the absence of an animal model, and therefore, information is derived solely from human studies, and understandably remains limited. Accordingly, development of strategies for case finding, diagnosis, and treatment are major foci of the ongoing KAEP.[4] As the outcome of VL having a PKDL sequel is determined by a complex interplay of parasite characteristics, vector biology, and host factors, with immune responses taking center-stage, development of diagnostic and therapeutic strategies requires an understanding of the host immune responses so that informed decisions are made.[9]

PKDL has different clinical manifestations, but not necessarily all the forms are present in every patient – (i) polymorphic – presence of a mixture of papules, nodules, and/or macules on the body – and (ii) macular – presence of hypopigmented patches which start as minute pin-point lesions and gradually expand in size and coalesce together to form large figurate lesions with islands of uninvolved skin.[10] Recent studies have indicated that differences exist in terms of immunopathology between these two clinical forms, which indicate possible differences in host–pathogen interactions and differential response to current anti-leishmanial strategies.[11,12] Accordingly, understanding the immune responses generated during different stages of VL and PKDL as well as in the different forms of PKDL is essential for the development of targeted interventions to prevent or treat PKDL effectively. This review traces the immune landscape of South Asian PKDL, highlighting the current knowledge of the immune responses underlying disease development and progression.

SEARCH STRATEGY

The electronic database PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) was searched for articles published till date (September 2024) using the terms "PKDL,," "post kala azar dermal leishmaniasis." Relevant articles from the authors' personal files were identified, and from the articles, relevant information had been included.

INNATE IMMUNE RESPONSES IN PKDL

Host innate immune responses are usually initiated at the time of pathogen entry. However, since PKDL is a chronic skin infection and the progression of VL to PKDL is still unclear, it is difficult to analyze the initial immunopathological changes in data derived from clinical samples. Although macrophages are the primary host cell for *Leishmania* parasites, neutrophils and dendritic cells (DCs) that are recruited to the infection site play important and distinct roles in shaping the immune response generated during the course of disease progression in PKDL.

NEUTROPHILS IN PKDL

In leishmaniasis, depending on the parasite species, neutrophils are proposed to play a dual role, being the conventional initial responders to infection, as also being the initial reservoirs for parasites.[13] Although neutrophils can effectively eliminate parasites, some *Leishmania* species have evolved distinct strategies to evade destruction and survive.[14] In addition, neutrophils at the infection site can influence the local skin micro-environment by rapidly releasing cytokines, thereby shaping the subsequent adaptive immune responses.[14] The role of neutrophils

in early stages of establishment of *Leishmania* infection is well-characterized, wherein they function as "Trojan Horses," and thereby safely transfer parasites to host macrophages.[15,16] In the chronic stages of *Leishmania* infection, such as in patients with PKDL, an increased presence of activated $CD66b^+$ neutrophils at the lesional sites has been observed, indicative of their presence beyond the acute stage of infection.[17] These infiltrated neutrophils are functionally active, as corroborated by their expression of CD64 (activation marker), myeloperoxidase (degranulation marker), and neutrophil elastase.[17] Furthermore, elevated plasma levels of neutrophil chemo-attractants (CXCL8/1/2/5, CCL2, and 20), along with increased levels of circulating and lesional IL-8, and enhanced lesional expression of IL-10 and IL-17A, are possibly involved in coordinating the recruitment of neutrophils to the lesional sites.[17] In addition, the increased levels of circulatory and lesional matrix metalloproteases MMP9 (released from specific/secondary granules of neutrophils) in PKDL, coupled with reduced collagen I, may translate into the disruption of matrix integrity, which could alter the lesional micro-environment and facilitate migration of immune cells.[17]

An interesting phenomenon, neutrophil "transdifferentiation" involves the acquisition of dendritic cell-like features (neutrophil–dendritic cell hybrids, N-DCs), challenging their traditional role as solely phagocytic cells.[18-20] This phenomenon in PKDL demonstrates acquisition of CD83 (a DC maturation marker) in circulatory neutrophils but is not accompanied by any alteration in the expression profile of antigen presentation (HLA-DR) and co-stimulation markers (CD80/86) [Mitali Chatterjee, personal communication]. In PKDL, elevated circulatory levels of TNF-α and IFN-γ endorse the expression of CD83 in neutrophils, while no significant change in levels of GM-CSF endorse the lack of conventional N-DCs with antigen presentation capabilities, leading to the formation of a unique subset of nonclassical N-DCs ($CD83^+$ neutrophils).[18] To delineate their functional role, if any, *L. donovani*-infected neutrophils exhibited expression of CD83, indicating formation of nonclassical N-DCs. Importantly, this was associated with increased generation of reactive oxygen species, heightened phagocytic capabilities, and apoptotic potential, suggesting that infection with virulent *Leishmania* triggered neutrophil transdifferentiation (Mitali Chatterjee, personal communication). This transformation possibly facilitated parasite uptake and their transfer to macrophages via the "Trojan Horse" mechanism. Taken together, in the absence of an animal model of PKDL, the contribution of neutrophils in the immunopathogenesis of PKDL still remains open-ended and conjectural, emphasizing the need for further research.

DENDRITIC CELLS IN PKDL

DCs are a diverse group of hematopoietic cells originating from the bone marrow and are distributed widely across various tissues. In the skin, they are regarded as the most efficient and specialized antigen-presenting cells, with a primary function in capturing, processing, and presenting antigens to T cells.[21] Dermal DCs comprise at least three distinct subpopulations, namely, (a) epidermal Langerhans cells (LCs) and (b) two types of migratory dermal DC (dDCs) subsets, which are distinguishable by their CD1a expression. DCs play a critical role in the immune response against *Leishmania* infection, particularly in Leishmaniasis. Their ability for initiating and modulating immune responses by capturing and presenting *Leishmania* antigens to T cells and subsequent activation of these naive T cells promotes adaptive immunity.[22] Accordingly, a central role for DCs in the dermis in modulating immune responses in Leishmaniasis has been proposed.[22] There was a marked reduction in LCs and dDCs at the lesional sites of PKDL, indicated by a decreased expression of CD1a and no evidence of an epithelioid granuloma formation.[23,24] This reduction in CD1a expression is not limited to specific downregulation of activated LC/dDCs as there is a concurrent decline in MHC class II and mRNA expression of IL-12p40 and is accompanied by an increase in IL-10 levels, suggesting an overall attenuation of LC/dDC functions.[24] Therefore, in PKDL, which is a chronic manifestation of the disease, the DC functions can become impaired due to the parasite's immune evasion mechanisms, leading to an ineffective antigen presentation.

Histopathological findings can vary widely in PKDL, akin to leprosy, where presentations depend significantly on clinical morphology, including focal to dense infiltrates in the dermis as well as tuberculoid/lepromatous granulomas in a minor proportion of PKDL cases.[25] With regard to DCs, both the polymorphic and macular variants of PKDL exhibited a reduction in LCs and dDCs at the lesional sites, although the decrease is more pronounced in polymorphic lesions.[11] Additionally, the HLA-DR expression was reduced in both PKDL forms,[11,24] similar to African PKDL, where epidermal LCs are depleted along with loss of dendritic morphology and HLA-DR expression.[23,26] In the dermis, these cells express B7-2 but not B7-1, and as LCs are key initiators of the skin's immune response, their reduction, along with loss of dendritic morphology, collectively indicated their impaired ability for antigen presentation, contributing to immunosuppression. In

this light, it is plausible that these changes are related to the immunosuppressive effects of UV-B rays, which are known to damage epidermal LCs and suppress contact hypersensitivity and allo-antigen responses.[27,28] Another interesting aspect is UV-B-induced damage to epidermal LCs,[27,28] that could possibly explain why their decrease is more significant in the polymorphic lesions, which primarily appear in sun-exposed areas,[11,28] *vis-a-vis* the macular form, where the hypopigmented patches are present even in photo-protected regions.[10] Conclusively, in Indian PKDL, the lack of granuloma formation, combined with the reduction of lesional DCs, results in immune suppression, which is crucial for parasite persistence and disease progression.[29]

MONOCYTES/MACROPHAGES IN PKDL

Monocytes/macrophages play a central role in the pathogenesis of leishmaniasis by serving both as host cells for *Leishmania* parasites and key mediators of the immune response.[30] The survival of *Leishmania* within host cells hinges on the parasite's ability to inhibit monocyte/macrophage activation and evade the host's immune response.[5,31] Although in PKDL the parasites are mainly restricted to dermal sites, alterations in the systemic cellular immunity have been reported,[29] wherein the circulatory monocytes exhibited a reduced expression of TLR-2 and 4, along with decreased generation of reactive oxygen and nitrogen species.[30] Collectively, this disrupted redox balance possibly accounts for the impaired antimicrobial functions of monocytes/macrophages, translating into enhanced parasite survival.

Macrophages typically function to eliminate pathogens through phagocytosis and activation of antimicrobial responses; typically, when exposed to Th1-associated cytokines, particularly IFN-γ, monocytes/macrophages adopt a heightened antimicrobial response against intracellular pathogens, known as the classically activated/M1 phenotype.[32] In PKDL, these cells are often skewed toward an immunosuppressive or alternatively activated M2 phenotype[30] that is usually associated with an increased proportion of Th2 cytokines such as IL-4, IL-13, IL-10, IL-33, and TGF-β.[32] Concerning disease presentation in PKDL, elevated mRNA levels of classical M2 markers such as *CD206, ARG1,* and *PPARG* in both monocytes and lesional macrophages suggested a shift toward M2 polarization phenotype, which was further supported by an increase in $CD68^+$ macrophages in dermal lesions and localization of arginase-1 and mannose receptor (CD206) within $CD68^+$ macrophages.[30] Additionally, altered vitamin D signaling is a key characteristic of PKDL, with increased plasma levels of 1α,25-dihydroxyvitamin D3 (1,25-D3) and upregulation of vitamin D3-associated genes [*VDR* (responsible for nuclear signaling of 1,25-D3), *CYP27B1* (encoding vitamin D-1α-hydroxylase which converts inactive prohormone to its bioactive 1,25-D3 form), and *LL-37* (a downstream antimicrobial effector peptide cathelecidin of the vitamin D signaling pathway)], further indicating M2 polarization.[30] Taken together, these findings suggest that in PKDL, the monocyte/macrophage subsets shifted toward an alternatively activated M2 phenotype, and this state impairs the macrophages' ability to effectively kill the parasites, promoting disease persistence. Importantly, anti-leishmanial treatment agents such as miltefosine leads to repolarization of monocytes to an M1 phenotype, suggesting that shifting from an M2 to M1 state can be a potential therapeutic strategy for PKDL, warranting further pharmacological exploration.[30,33]

Major monocyte/macrophage specific chemo-attractants such as CCL2 and CCL7 had elevated circulatory levels in patients with PKDL, thereby facilitating dermal migration of monocyte/macrophages.[11] Polymorphic PKDL demonstrated a significantly higher proportion of lesional $CD68^+$ macrophages compared to the macular group;[11] however, differences, if any, in their polarization status between the two clinical forms need to be warranted. The lower degree of infiltration of monocytes/macrophages at the lesional site in macular PKDL may also have an impact on the lesional concentration of liposomal amphotericin B (LAmB), as validated in a murine model of CL.[34] This was supported by a study which reported incomplete elimination of parasites in PKDL cases treated with LAmB, more significantly in the macular variant.[12] Therefore, it could be concluded that the dramatic decrease in the parasite burden in the polymorphic vs. macular variant could be due to a higher accumulation of LAmB, secondary to a higher infiltration of $CD68^+$ macrophages, suggesting pharmacokinetic differences between the two forms of PKDL and emphasizing the need for patient stratification.

CELL-MEDIATED AND HUMORAL IMMUNE RESPONSES IN PKDL

The interaction between the parasite and host cells ultimately determines the infection course and the disease outcomes of human leishmaniasis. The resolution of infection is attributed to the establishment of cell-mediated immunity, specifically the activation and differentiation of T lymphocytes that stimulate the production of cytokines, which induce the activation of infected mononuclear phagocytes and culminate with parasite elimination.[35] Furthermore, in terms of humoral

immunity, B cells may also play an important role in *Leishmania* infection, although they have been neglected for a long time.[36]

T CELLS IN PKDL IMMUNOLOGY

The nature of T-cell responses often mirrors the disease outcome, and depending on their functional responses, they can be protective or pathogenic.[37] During skin infection with *L. donovani*, protection would be dependent on the induction of proper Th1, Th17, and $CD8^+$ cytotoxic T-cell-mediated responses. However, decreased generation of these protective immune responses, along with dominant responses of $CD4^+$ Treg cells, Th2, and regulatory $CD8^+$ T cells, has been proposed to be associated with the development of PKDL.[38]

In Indian PKDL, the concentrations of peripheral blood $CD3^+$ T lymphocytes, $CD3^+CD4^+$ Th cells, $CD3^+CD8^+$ T cytotoxic cells, $CD3^-CD56^+$ NK cells, $CD3^+CD56^+$ NKT cells, and $CD4^+CD25^+$ Treg cells were comparable to those of healthy individuals, and this was evident even after completion of treatment.[39] Although these patients exhibited intact lymphoproliferative responses to phytohemagglutinin, a nonspecific mitogen, evident by the comparable number of IFN-γ-, IL-2-, IL-4-, and IL-10-expressing lymphocytes, there was a significant increase in the percentage of IL-10–expressing $CD3^+CD8^+$ lymphocytes in response to *L. donovani* antigen.[39]

In Indian PKDL, there is a high infiltration of $CD3^+$ T lymphocytes at the lesional sites.[40] However, this population consisted mainly of $CD8^+$ T cells with a conspicuous absence of $CD4^+$ T cells, which may account for its inability to be a self-curing lesion.[11,41] The increased circulatory levels of Th2 chemo-attractants CCL17 and CCL22 were associated with enhanced expression of the receptor CCR4 at the lesional site, which facilitated the homing of $CD8^+$ T cells to the skin.[41] In the peripheral blood of PKDL patients, there was a significant decrease in the levels and expression of CD26 and CD45RO on $CD8^+$ T cells, along with decreased plasma levels of sCD26 and a concomitant increase in the proportion of $CD45RA^+CD8^+$ T cells, which possibly contributed to T-cell unresponsiveness.[42] Interestingly, the lesional $CD8^+$ T cells reported a reduced expression of cytotoxicity markers perforin, granzyme, and Zap-70, while the proportion of activated $CD8^+$ $CD127^+$ T cells was decreased in peripheral blood.[41] Additionally, the enhanced expression of programmed death-1 in peripheral blood and lesional skin indicated functional exhaustion, which possibly facilitated disease progression in Indian PKDL.[41]

Regulatory T cells (Tregs) act at the interface of host and pathogen interactions in human infectious diseases and are known to play a dual role. Therefore, they benefit the host by limiting immune-mediated pathology and also facilitate chronic pathogen persistence by reducing effector immunity and clearance of infection.[43] Treg cell subpopulations including natural Treg (nTreg) and induced Treg (iTreg) cells emerge in the thymus from immature precursors or in the peripheral lymphoid organs from stimulated naive $CD4^+$ T cells in the presence of TGF-β and IL-2, respectively.[44] The predominant Treg types are $CD4^+$ and express the surface IL-2Rα chain CD25 and/or the forkhead box transcription factor Foxp3.[45] In patients with PKDL, circulatory $CD8^+CD28^-$ and antigen-induced $IL\text{-}10^+CD3^+$ lymphocytes were increased at disease presentation and decreased with treatment.[40] Analysis of nTreg markers (Foxp3, CD25, and CTLA-4) and IL-10 demonstrated their elevated expression in the lesional skin of PKDL cases, with the expression being nodules > macules/papules.[40,46] Their mRNA expression correlated positively with parasite burden, suggesting their role in disease severity in PKDL.[46]

CYTOKINE MILIEU IN PKDL

Understanding the role of the T cell-related cytokine network in the pathogenesis of PKDL lesions provides insights concerning the role of each cytokine in disease protection or susceptibility [Table 1].[38] The local production of Th1 cell-related cytokines (including IFN-γ, TNF-α, and IL-12), Th17 cell-derived cytokines (such as IL-17 and IL-22), and $CD8^+$ cytotoxic T lymphocyte-derived IFN-γ can provide protective effects against PKDL.[38] However, there is also a dominant presence of regulatory T cell-derived cytokines (such as IL-10 and TGF-β), Th2 cell-derived cytokines (such as IL-4/IL-13), M2 macrophage-derived cytokines (such as IL-4 and IL-10), keratinocyte-derived IL-10, regulatory $CD8^+$ T cell-derived IL-10, and dendritic cell-derived IL-10, IL-27, and IL-21, which will enhance parasite persistence and PKDL development.[38] In PKDL, several studies have reported the presence of a mixed pro and anti-inflammatory cytokine milieu. Monocytes/macrophages in circulation have enhanced IL-12p40, IL-4, IL-10, and IL-13 levels,[30] along with lower amounts of IL-6, IL-1β, and IL-8.[39] At lesional sites, an increased mRNA expression of both pro and anti-inflammatory cytokines has been reported.[39,40,48,50,52,53] However, the concomitant decrease in the expressions of IFN-γR and TNF-αR1, perhaps mediated by high IL-6 levels, accounted for their inability to mediate the anticipated host protective parasiticidal action.[48,53] Furthermore, the increased mRNA expression of counter-regulatory

Table 1: Role of major cytokines in post kala-azar dermal leishmaniasis (PKDL)

Type of inflammatory milieu	Name of the cytokine	Functions	References
Pro-inflammatory (Th1 derived)	IFN-γ	Promotes host defense through activation of macrophages and $CD8^+$ cytotoxic T lymphocytes, essential in protection against PKDL but can also disrupt melanogenesis, leading to hypopigmentation	[40,47,48]
	TNF-α	Enhances inflammation and promotes parasiticidal activity; however, its receptor downregulation can impair host protective responses	[47,48]
	IL-8	Chemotactic cytokine that recruits neutrophils, increased expression in circulation and at lesional sites of patients with PKDL, and mediates recruitment of neutrophils to the dermis	[17,49]
	IL-6	Promotes inflammation but can also have regulatory effects by suppressing Th1 responses, contributing to PKDL pathogenesis	[30,39,48,50]
	IL-1β	Potentiates inflammatory responses; responsible for inflammasome activation at the lesional sites	[39,40,47]
	IL-12p40	Subunit of IL-12, enhanced production in PKDL, involved in promoting Th1 responses but also associated with regulatory functions in PKDL progression	[30]
Pro-inflammatory (Th17 derived)	IL-17	Promotes inflammation and has been associated with upregulation in PKDL lesions	[51]
	IL-21	Supports regulatory immune responses, promoting immunosuppressive conditions in PKDL lesions	[38]
	IL-23	Promotes Th17 cell differentiation and survival, linked to elevated levels in PKDL lesions	[51]
Regulatory cytokines	TGF-β	Suppresses Th1 and pro-inflammatory cytokines, promoting an immunosuppressive environment conducive to parasite persistence	[48,50,52]
	IL-10	Potent immunosuppressive cytokine produced by various cells (Tregs, keratinocytes, dendritic cells, etc.); inhibits Th1 responses and promotes parasite persistence in PKDL lesions	[30,39,41,46,48,50]
Anti-inflammatory (Th2 derived)	IL-4	Downregulates Th1 responses, promoting parasite persistence in PKDL	[30,48]
	IL-13	Works alongside IL-4 to suppress inflammatory responses, enhancing disease susceptibility	[30]
	IL-5	Produced by Th2 cells, maintains progression of $CCR4^+$ $CD8^+$ T cells	[41]

PKDL: post kala-azar dermal leishmaniasis, Tregs: regulatory T cells

cytokines, such as TGF-β and IL-10,[48,50] possibly curtailed the pro-inflammatory cytokine-based immune responses and instead supported an immunosuppressive milieu accounting for parasite persistence. Elevated plasma IL-17 levels and upregulation of Th17 cell-linked markers, for example, IL-17 and IL-23 and RORγt, have been reported in PKDL lesions.[51] The increased presence of pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-1β along with elevated levels of IFN-γ-inducible chemokines such as CXCL9/10/11 could hamper the melanogenesis signaling pathway within the melanocytes, thereby resulting in the destruction/dysfunction of melanocytes and leading to hypopigmentation, a feature exclusively observed in patients with PKDL.[47,54]

HUMORAL IMMUNE RESPONSES IN PKDL

Although studies have proposed the ability of both Th1 and Th2 cells to support B-cell responses, the latter is found to be more adept in this respect, as evident by their cytokines being important regulators of B-cell proliferation and differentiation.[55] In general, studies analyzing the humoral immune responses in patients with PKDL have demonstrated a high proportion of circulatory anti-leishmanial antibodies.[56-59] In terms of the immunoglobulin subtype and the clinical form of PKDL, there was a greater increase in the levels of Ig, IgM, and IgG in the polymorphic variant compared to its macular counterpart, with a significant decrease following treatment.[59] Furthermore, the most significantly altered IgG subclasses were IgG1 and IgG3, the scenario being more stronger in the polymorphic form.[59] These differences could be attributed simply to the higher parasite load observed in polymorphic PKDL which is also supported by higher IgG avidity[57,60] or variations in host immune responses, a question which is pertinent, but remains unexplored.

In Indian PKDL, the proportion of circulating $CD19^+$ B cells are decreased[57,61] whereas, in dermal lesions of both variants, an increased expression of $CD20^+$ B cells was demonstrated.[11] However, within the circulatory $CD19^+$ B-cell population, there was a significantly increased proportion of switched memory B cells ($CD19^+IgD^-CD27^+$) and plasma cells ($CD19^+IgD^-CD38^+CD27^+$). The increased levels of circulatory B-cell chemo-attractants such as CCL20 and CXCL13 and absence of lesional Ki67 expression accounted for the higher expression of $CD20^+$ B cells and $CD138^+$ plasma cells along with IgG in the lesional skin, which can facilitate parasite entry and disease progression.[61]

CONCLUSION

Despite considerable research efforts, there still exists a lack of an animal model to fully grasp the natural course of PKDL progression. It is also important to consider that the current understanding of this disease is derived mainly from observations made after the infection is well-established, providing predominantly a chronic perspective, and the acute phase of the infection till date

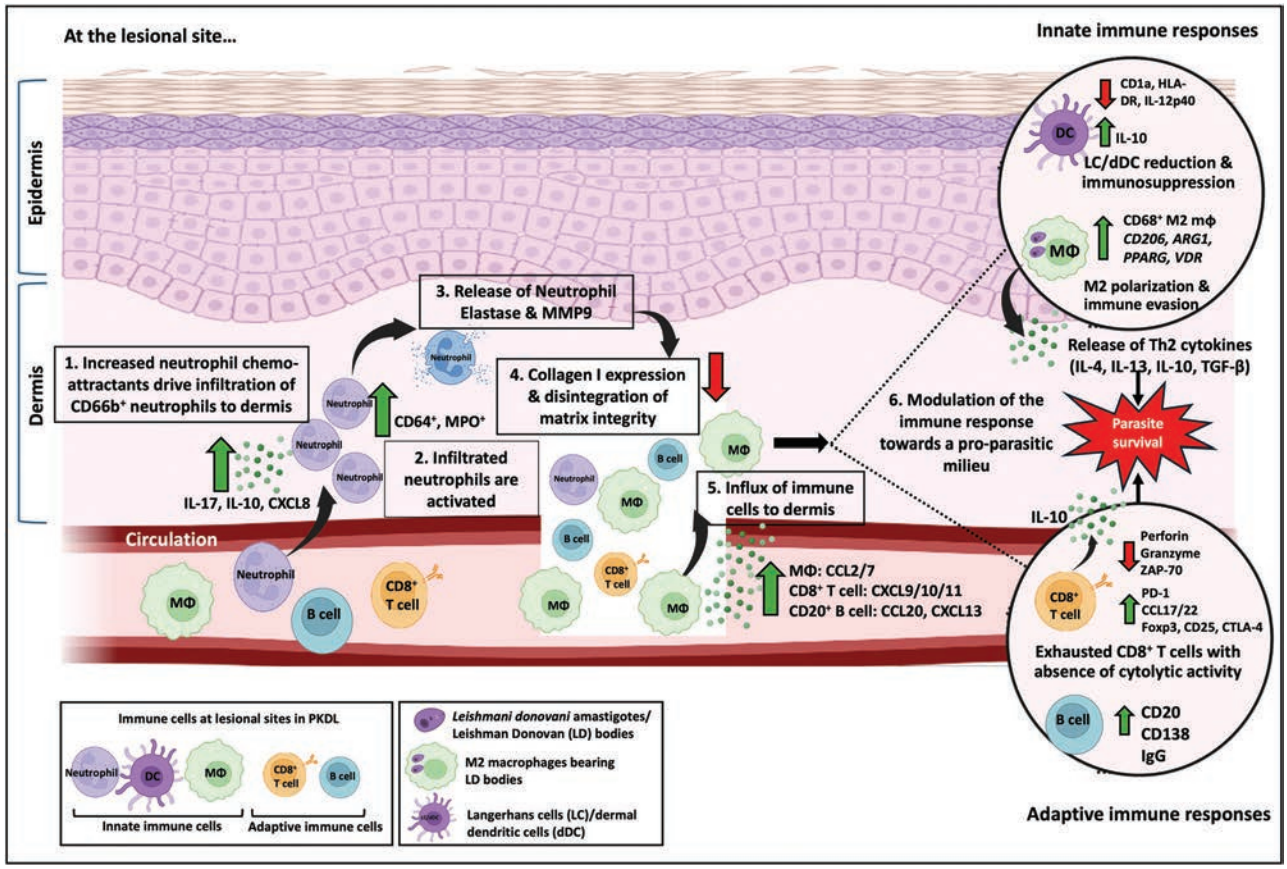

Figure 1: The involvement of innate and adaptive immune cells in disease pathophysiology of South Asian post kala-azar dermal leishmaniasis (PKDL). In patients with PKDL, increased levels of neutrophil chemo-attractants (e.g., IL-8/CXCL8) and cytokines (e.g., IL-17, and IL-10) facilitate recruitment of neutrophils to the lesional skin (1). These neutrophils are activated in terms of their expression of myeloperoxidase and CD64 (2). This results in the increased secretion of neutrophil elastase and matrix metalloproteinase 9 (3), which in turn leads to decreased expression of extracellular matrix protein collagen-1 and disintegration of tissue microarchitecture (4). As a result, immune cells (e.g., monocyte/macrophages, dendritic cells (DCs), T cells, and B cells) are homed from the circulation to the dermal site, aided by increased levels of circulatory chemokines specific for each immune cell (5). At the lesional site, these recruited innate (DCs and macrophages) and adaptive (CD8⁺ T cells and B cells) immune cells modulate the immune landscape (6), which ultimately leads to parasite sustenance and disease maintenance

remains largely elusive and cannot be fully characterized. Therefore, what remains unanswered till date is the precise mechanisms of pathogenesis driving the onset of PKDL. It has been hypothesized that PKDL may present as an immune reconstitution inflammatory syndrome in VL patients following completion of treatment, leading to a loss of immune suppression. In this study, we have summarized recent advances in our understanding of the immune responses generated in South Asian PKDL and, when possible, integrated our knowledge regarding any similarities/dissimilarities between its different clinical forms. Although information is still fragmentary, evidences indicate the presence of an enhanced Th1/Th2 immune milieu in PKDL, skewed more toward the Th2 phenotype, which creates an immunosuppressive environment that allows parasite survival; however, the underlying cause of Th1 immune failure in the skin following VL treatment, coupled with the sustained Th2 response, continues to be a topic of active debate. It is noteworthy that African PKDL represents an immunological response that is typically self-limiting, whereas Indian PKDL often manifests as a recrudescence of infection; therefore, further research is warranted to investigate the potential reasons for the differing immune responses in Indian PKDL compared to those observed in African PKDL.

In response to *L. donovani*, the host mounts an immune response, and an array of immune cells infiltrate at the lesional sites in PKDL, and the conflict between the host and parasite begins to gain the ground [Figure 1]. In the host's effort to combat the infection, both innate and adaptive immune cells are recruited at the lesional sites. The infiltrated neutrophils are activated, but their limited numbers result in an ineffective parasite clearance; instead, they contribute more significantly to disruption of matrix integrity, thereby facilitating the infiltration of other immune cells into the dermis. As immune cell accumulation intensifies at the site of infection, the parasites activate their

survival mode and cleverly modulate immune responses to favor their persistence. This is characterized by an increased population of antigen-specific IL-10-producing anergic T cells, a decreased presence of DCs at the lesional sites, and a substantial infiltration of $CD68^+$ alternatively activated M2 macrophages, along with the depletion of $CD4^+$ T cells and exhaustion of dermal $CD8^+$ T cells, which individually or collectively contribute to an immunosuppressive environment supporting parasite survival. These immune evasion strategies employed by *Leishmania* highlight the critical need for innovative immunotherapeutic interventions aimed at restoring effective immune function so as to meet the end goal of elimination by 2030.

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Conflict of interest

There are no conflicts of interest.

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Review Article

Antirelapse therapy for visceral leishmaniasis in immunocompetent population and diagnostic dilemmas in therapeutic assessment in India

Rishikesh Kumar, Krishna Pandey

Indian Council of Medical Research-Rajendra Memorial Research Institute of Medical Sciences, Patna, Bihar, India

Abstract

Visceral leishmaniasis (VL), a severe parasitic infection caused by *Leishmania* species, presents a significant challenge due to its potential for relapse despite treatment. This review aims to rationalize antirelapse therapies for VL and address diagnostic dilemmas encountered in therapeutic assessment. By evaluating current treatment strategies, examining relapse mechanisms, and highlighting diagnostic challenges, we aim to provide a comprehensive understanding of effective management and assessment of VL relapse.

Keywords: Antirelapse therapies, immunocompetent VL, kala-azar, therapeutic assessment, visceral leishmaniasis

Address for correspondence: Dr. Krishna Pandey, Indian Council for Medical Research-Rajendra Memorial Research Institute of Medical Sciences, Agamkuan, Patna 800007, Bihar, India.
E-mail: drkrishnapandey@yahoo.com
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BACKGROUND

Visceral leishmaniasis (VL), or kala-azar, is a systemic parasitic disease with a high fatality potential in untreated cases.[1] It is transmitted by the bites of infected female sandflies of *Phlebotomus* species and is characterized by hepatomegaly, splenomegaly, irregular bouts of fever, and anemia.[1,2] With only 520 VL cases in 2023, India has achieved the elimination target that is, less than one case per 10,000 population at the block level (National Center for Vector Borne Diseases Control, India).[3] In its post-elimination phase, it is required to sustain the elimination status for three consecutive years for the certification for VL elimination by the World Health Organization (WHO). Recurring or relapsing VL cases can potentially jeopardize the hitherto gains achieved in VL elimination as it may lead to continued VL transmission through competent vectors.

There is no known pathophysiologic distinction for the characterization of a relapse in VL and, therefore, a relapse remains a synonym of a recurrence in VL and is used interchangeably. VL relapses can be a function of increased parasitic tolerance for antileishmanials, suboptimal dosing/bioavailability of antileishmanials, compromised immunity, and a surmised ability of the parasite to transform into a latent form for which employed antileishmanials remain ineffective.[4-8] Mechanisms of VL relapse are summarized in Table 1. Besides, a reinfection is regarded as a relapse in routine clinical settings. However, the significance of distinguishing a case of relapse from a case of reinfection is that the former may indicate a failing drug regimen.

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The estimates of VL relapse may vary remarkably depending on the duration of post-treatment follow-up, the laboratory tests used for the detection of VL infection, and the definition of relapse.[9,10] If the rate of relapse with a given antileishmanial goes up to 10% or more it would not be considered an effective drug regimen in the respective region/setting. Therefore, it is of paramount importance to evaluate the efficacy of antileishmanials, intermittently. Liposomal amphotericin B (LAmB) in a single dose at 10 mg/kg body weight was first recommended for the treatment of VL in the Indian subcontinent in the year 2010.[11] However, miltefosine remained the first-line treatment regimen in the elimination program for VL in India until 2014 and was replaced by the single dose LAmB (10 mg/kg body weight), thereafter,[6] and it has been standing as the first-line treatment for VL in India. The rate of VL relapse in the Indian subcontinent up to 6 months after a single dose of LAmB has been estimated as 4.5% (95% confidence interval = 2.6–7.5) during 1983–2021.[9] The relapse rate of VL was reported as 1.4% with a high dose of LAmB (20 mg/kg) in non-human immunodeficiency virus (HIV) patients during 2007–2012 (median = 10.1 months) in a cohort study from Bihar, India.[12] In another study from Bihar, the relapse rate with single dose LAmB (10 mg/kg) was 3% up to 6 months and 5.8% by 12 months of post-treatment follow-up in HIV-negative VL patients during 2013–2017.[13] In a study in West Bengal, among 74 cases treated with a single dose of LAmB (10 mg/kg), ~7% relapsed by 2 months post-treatment.[14] Many contemporary experts on VL case management in India and abroad suggested either increasing the dose of LAmB from 10 mg/kg body weight to a higher dose or using it in combination with other antileishmanials for treating VL relapses (WHO, GDG meeting on VL and PKDL treatment, Monday, May 6, 2024). Nonetheless, there may be other factors related to the host and parasite having an association with VL relapse. The risk factors associated with VL relapse remain a seldom less explored aspect of VL research in immunocompetent populations. Despite advances in treatment, relapse remains a common issue, complicating disease management and patient outcomes. As of now, there does not exist a guideline for the clinical management of HIV-negative relapsing VL patients. The most commonly practiced and/or advised treatments are summarized in Table 2.

Table 1: Mechanisms of relapse in visceral leishmaniasis

1.Inadequate initial therapy	Incomplete or insufficient treatment may fail to eradicate the parasites completely, leading to relapse. Factors influencing treatment adequacy include drug dosage, duration, and patient adherence
2.Drug resistance	Resistance to first-line treatment, such as pentavalent antimonials such as sodium stibogluconate, has emerged as a significant concern. Resistance can lead to therapeutic failure and increase the risk of relapse
3.Host factors	Immunosuppression, whether due to human immunodeficiency virus co-infection or other factors, can impair the host's ability to clear the infection, increasing the likelihood of relapse. Host genetic factors may also contribute to relapse by affecting response to therapy
4.Parasitic factors	Genetic variations and adaptations in *Leishmania* species may contribute to relapse by affecting parasite survival and replication in the host

Diagnostic specimens used for VL before and after treatment completion remain mismatched. Splenomegaly and hemostatic propensity together guide the selection or rejection of splenic puncture for the collection of diagnostic specimens from a suspected or treated VL patient. A confirmatory diagnosis of VL rests upon microscopic or nucleic acid amplification methods for specimens such as splenic or bone marrow aspirates in the Indian subcontinent.[15] The procedure of splenic aspiration poses a relatively higher threat of untoward occurrences than bone marrow aspiration. Nonetheless, a splenic aspirate is known to be a better diagnostic specimen for VL (high positive predictivity) in comparison with

Table 2: Current anti-elapse therapies for immunocompetent visceral leishmaniasis (VL) patients (reference: https://ncvbdc.mohfw.gov.in/WriteReadData/l892s/opertional-guideline-KA-2015.pdf)

1.Liposomal amphotericin B (LAmB)	LAmB is the preferred treatment for relapsed VL due to its high efficacy and safety profile. The regimen typically involves 3–5 mg/kg body weight administered intravenously over 3–5 days. Its advantages include reduced nephrotoxicity and broad-spectrum activity against *Leishmania* species
2.Pentavalent antimonials	While traditionally used, pentavalent antimonials such as sodium stibogluconate are less effective for relapsed cases due to resistance issues. These drugs may still be considered in specific scenarios based on local resistance patterns and treatment history
3.Miltefosine	Miltefosine offers an oral alternative for relapsed VL. The standard regimen involves 50 mg twice daily for adults and 2.5 mg/kg body weight daily for children, administered for 28 days. It is effective and has the benefit of oral administration, facilitating outpatient treatment. Side effects of miltefosine include nausea, vomiting, diarrhea, and abdominal discomfort. Moreover, miltefosine is contraindicated during pregnancy and women of childbearing age should use contraception for 3 months after treatment
4. Paromomycin	Paromomycin, administered intramuscularly, is an option for relapsed VL with a regimen of 11 mg/kg body weight once daily for 21 days. It is useful in cases where other treatments are unavailable or unsuitable. Although therapeutic trials of this drug were conducted in India, currently, use of this drug is limited by its availability in India
5. Combination therapy	Combination therapy, such as LAmB with miltefosine should be considered based on patient-specific factors and regional guidelines. Other effective combinations of LAmB or miltefosine with paromomycin are limited by the availability of the latter in India. Advantage of combination therapy includes a shorter treatment regimen with miltefosine

a bone marrow aspirate. Therefore, a splenic aspirate is obtained in patients with splenomegaly who are fit and suspected of VL at baseline. However, splenomegaly subsides following antileishmanial medication in most patients. Consequently, a bone marrow aspirate is used for assessing the antileishmanial treatment efficacy in all such cases. This mismatch of the diagnostic specimen at pre- and post-treatment assessment and its impact on the measures of therapeutic outcomes in VL remains unaddressed. Therefore, it stands to reason that the therapeutic efficacy assessment using an inferior diagnostic specimen would result in a false overestimation of the therapeutic success.

Furthermore, laboratory approaches used for diagnosing VL such as microscopy, serology, culture, and nucleic acid amplification tests are heterogeneous as the employed specimen matrix and/or diagnostic protocol differ by region, level of health care centers, and investigators. For example, venous blood is not used in routine microscopy for VL in India but, a buffy coat separated from venous blood is used for routine microscopic evaluation for VL in Bangladesh.[16] Similarly, isolation of genomic deoxyribonucleic acid differs from using bone marrow, splenic, or lymphatic aspirates to buffy coat or liver biopsy for the detection of *Leishmania* species.[15,17] Moreover, despite the superior performance of nucleic acid amplification tests in diagnosing VL [end-point polymerase chain reaction (PCR), quantitative PCR, loop-mediated isothermal amplification, and recombinase polymerase amplification.), adaptability, and recognition of one or more of these tests as "reference test" is limited by their cost, sophistication/complexity, and more importantly lack of a standard protocol. Understanding the rationale behind antirelapse therapy and addressing diagnostic challenges is crucial for improving therapeutic strategies and patient care.

SEARCH STRATEGY

The electronic database PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) was searched for articles published to date (September 2024) using the terms "VL" and "visceral leishmaniasis" or "kala-azar" and "Relapse" or "recurrence" and "immunocompetent" or "HIV-negative." Relevant articles from the authors' personal files were identified, and from the articles, relevant information was included.

DIAGNOSTIC DILEMMAS IN THERAPEUTIC ASSESSMENT

Detection of relapse

Accurate detection of relapse is challenging due to the limitations of current diagnostic tests. Conventional methods include bone marrow or splenic aspiration, but these can be invasive and may not always detect low-level parasitemia.

Limitations of diagnostic tests

Microscopy

While useful, microscopy may have limited sensitivity, particularly in detecting low parasite loads.

Serology

Serological tests may remain positive long after successful treatment, complicating the distinction between active relapse and residual immune response, for example, the rk39 immunochromatographic test.

Polymerase chain reaction

PCR offers high sensitivity and specificity but may not be universally available or practical in all settings.

Monitoring treatment response

Evaluating treatment response involves monitoring clinical improvement and laboratory parameters. However, there is no standardized approach, and interpretation can be affected by the timing of follow-up assessments and the variability of clinical responses.

Addressing co-morbid conditions

Co-morbid conditions, such as HIV, complicate therapeutic assessment and increase the risk of relapse. Integrated management of VL and co-existing conditions is essential for accurate diagnosis and effective treatment.

RECOMMENDATIONS FOR IMPROVED MANAGEMENT

Enhancing diagnostic capabilities

Developing rapid tests

Investing in rapid diagnostic tests with high sensitivity and specificity can improve early detection and monitoring of VL relapse. The rk39 immunochromatographic test is one such rapid test.

Implementing molecular methods

Expanding the use of PCR and other molecular techniques in endemic regions can enhance diagnostic accuracy.

Optimizing treatment regimens

Individualized treatment plans

Tailoring treatment based on individual patient factors, including drug resistance patterns and co-morbidities, can improve outcomes.

Exploring combination therapies

Utilizing combination therapies and new drug formulations may offer more effective solutions for relapsed VL.

Improving access to care

Increasing drug availability

Ensuring access to effective treatments, particularly in resource-limited settings, is crucial for managing relapse and improving patient outcomes.

Strengthening healthcare infrastructure

Enhancing healthcare infrastructure and training for healthcare providers can facilitate better management and assessment of VL.

CONCLUSION

Relapse in VL poses significant challenges to treatment and management. Rationalizing antirelapse therapies involves understanding the mechanisms of relapse and selecting appropriate treatment regimens based on current evidence and resistance patterns. Addressing diagnostic dilemmas through improved detection methods and monitoring strategies is crucial for effective therapeutic assessment. By advancing research, optimizing treatment approaches, and improving diagnostic capabilities, we can enhance the management of VL and reduce the burden of relapse.

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Conflicts of interest

There are no conflicts of interest.

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Review Article

Advancements in diagnostics for visceral leishmaniasis: Current landscape and future directions

Mudsser Azam[1], Bharti Singhal[1], Ruchi Singh[1,2]

[1]Indian Council of Medical Research-National Institute of Child Health and Development Research, Safdarjung Hospital Campus, [2]Indian Council of Medical Research, New Delhi, India

Abstract

Visceral leishmaniasis (VL) is a significant global health concern, causing considerable morbidity and mortality. The prevalence of VL is influenced by environmental conditions and limited access to healthcare services. Accurate diagnostics are crucial for monitoring and controlling disease spread. This review examines current advancements in diagnostic technologies for VL, focusing on the need for highly sensitive, easily applicable, and affordable diagnostics. Parasitological examination, serological tests, molecular assays, and rapid diagnostic tests are discussed in detail, highlighting their strengths and limitations. Furthermore, we have discussed global efforts and initiatives aimed at improving diagnostics and conclude by exploring future directions in the field. Improved diagnostic capability, effective treatment regimes, and comprehensive public health initiatives are essential to reduce the global burden of VL. Furthermore, research and development are needed to enhance diagnostic tools for VL, particularly in resource-poor endemic countries.

Keywords: Diagnosis, rapid test, visceral leishmaniasis

Address for correspondence: Dr. Ruchi Singh, Scientist F, Discovery Research Division, Indian Council of Medical Research, Ansari Nagar, New Delhi 110029, India.
E-mail: ruchisingh.nip@gov.in; ruchisp@gmail.com
Submitted: 18-Oct-2024, **Accepted:** 28-Oct-2024, **Published:** XX-XX-XXXX.

INTRODUCTION

Visceral leishmaniasis (VL), also called kala-azar, is caused by a protozoan parasite, *Leishmania donovani*. VL circulates globally, impacting 90 countries and putting over 350 million people at risk of infection. VL is endemic in several parts of the world including South Asia—widespread epidemics have been reported from Bangladesh, Bhutan, India, and Nepal.[1] In East Africa, the most affected countries include Kenya, Ethiopia, Somalia, and Sudan;[2] in South America, countries with high incidences of VL are Bolivia, Peru, and Brazil;[3] and from the Mediterranean Basin, cases have been documented from parts of southern Europe and the Middle East.[4]

Annually, an estimated 50,000 to 90,000 new cases arise, with merely 25%–45% reported to the World Health Organization (WHO). These proportions are likely underestimates as *Leishmania* is not always notifiable and treatment is not always sought due to cost or geographic constraints.[4,5] In 2022, more than 90% of new cases reported to WHO occurred in 13 countries; Brazil, China, Eritrea, Ethiopia, India, Iraq, Kenya, Nepal, South Sudan, Somalia, Sudan, Yemen, and Uganda.[6]

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The *Leishmania* parasites can be transmitted through the bite of infected female phlebotomine sandflies.[7] The dispersion of VL is affected by environmental conditions, including temperature and the presence of appropriate sandfly vectors. The disease is frequently found in areas with inadequate housing, insufficient health facilities, and limited access to health services. VL primarily affects the internal organs, predominantly the liver, spleen, and bone marrow, leading to several signs and symptoms that include anemia, weight loss, long-standing fever, and hepatosplenomegaly, if not treated, it may lead to death.[8,9]

Untreated VL represents a serious health threat with a high mortality rate and results in approximately 20,000–40,000 deaths per year.[10] The failure of vital organs and infection can lead to serious complications.[9] VL burdens health services, especially in resource-poor endemic countries where special facilities are needed.[11] Leishmaniasis affects individuals across all age groups; however, there is a higher prevalence among males and elderly individuals.[12,13] VL also imposes a significant financial burden on families and communities due to therapy costs and potential social isolation and psychological distress.

Asymptomatic and paucisymptomatic infections pose a significant challenge, as undiagnosed patients can unknowingly spread the disease, contributing to disease transmission.[14,15] Screening studies, including those involving blood donors, have found asymptomatic infections, indicating that they might be more frequent than symptomatic ones.[16,17]

The number of reported kala-azar cases in the three endemic countries, that is, India, Bangladesh, and Nepal, has declined by 96.2% from 2007 to 2021. By the end of 2021, 99% of the implementation units in the Indian subcontinent achieved the elimination target, with only 1% remaining above the elimination threshold.[18] Bangladesh has attained the kala-azar elimination phase. India has reported significant progress in the direction illustrating the role of an appropriate programmatic approach. The global burden of VL can be reduced through improved diagnostic capability, effective treatment regimes, and comprehensive public health initiatives.[19] WHO highlighted the importance of ultrasensitive and rapid diagnostic tests (RDTs) as essential tools for identifying asymptomatic infections and cases of co-infection to ensure accurate diagnosis. This review focuses on the current advancements in diagnostics for VL as highly sensitive, easily applicable, and affordable diagnostics are required to monitor the disease prevalence.

ADVANCEMENTS IN DIAGNOSTIC TECHNOLOGIES

Parasitological examination

The conventional diagnosis is based on the microscopic analysis of amastigotes in tissue aspirates from several organs, including the liver, spleen, lymph nodes, and skin. For VL, microscopy has a sensitivity of 93%–99% for spleen aspirate, 53%–86% for bone marrow, and 53%–65% for lymph node aspirates, whereas for post-kala-azar dermal leishmaniasis (PKDL) it has a sensitivity of 67%–100% for nodular lesions, 36%–69% for papular lesions, and 7%–33% for macular lesions.[20,21] For VL, using more invasive specimens increases microscopy sensitivity.[22] However, it has limitations in identifying the genus level and cannot be used to differentiate species because all *Leishmania* species are morphologically very similar.[23]

Parasite isolation by culturing the tissue aspirate is a vital method used for diagnosing leishmaniasis. Using the newer microcapillary method, the culture of *Leishmania* promastigotes resulted in an increase in sensitivity from 69.2% with traditional culture methods to 92.3%, with a slight change in specificity (98.9%–97.8%), respectively.[24] However, the culture method is time-consuming, costly, and challenging, making it impractical in many clinical settings.

Machine learning (ML) has recently been used in the microscopic examination of leishmaniasis, relying on image quality and specific algorithms.[25] The Viola–Jones algorithm, the ML technique for object detection, achieved a 70% accuracy in identifying individual parasites, whereas detecting infected macrophages resulted in a 60% accuracy.[25]

Serological tests

In VL, the host-parasite interaction induces hyperimmunoglobulinemia, which is extensively used for diagnosis. Standard serological assays (antibody detection based) used for diagnosing VL include the enzyme-linked immunosorbent assay (ELISA),[26,27] western blot, immunoblotting,[28] direct agglutination (DAT),[29] latex agglutination test (LAT),[30] and immunofluorescence antibody test.[22]

DAT involves the agglutination of promastigotes in conjunction with anti-leishmanial antibodies.[28] It is a simple, reliable, cost-effective, and partially quantitative test that has been approved in various countries, including India, Brazil, Nepal, Sudan, Bangladesh, Kenya, and Ethiopia. The primary limitation of the DAT is its lack of prognostic value

for determining disease cure, as well as its time-consuming and labor-intensive nature.

Fluorescent antibody tests (FATs) can be either direct, in which the labeled antibody binds to the antigen, or indirect, in which a secondary polyclonal antibody binds to the patient's generated antigen.[31] To minimize the cross-reactions encountered in FAT with trypanosomal sera, now promastigote is used as an antigen.[28]

The sensitivity of ELISA is modulated by the antigen used to capture a specific antibody. For instance, the commonly used crude soluble antigen offers sensitivities ranging from 80% to 100%. However, cross-reactions with trypanosomiasis, tuberculosis, and toxoplasmosis have been observed.[32] Aronson *et al.* assessed new immunogenic screening; and identified antigens (antigenic *Leishmania infantum* peptides) based on linear B cell epitopes that can improve the sensitivity and specificity of VL serodiagnosis in immunoassays using single or multiple antigens.[33]

The Leishmanin or Montenegro skin test consists of an intradermal injection of antigen. A positive result is indicated by an induration of 5 mm or more. The sensitivity and specificity with this cutoff point have been reported as 97.4% and 93.9%, respectively.[34] It is highly sensitive in cutaneous leishmaniasis (CL), detecting past and active cases. However, in active VL, patients may not react due to anergy, reflecting only past exposure.[35]

Immunochromatographic RDTs have significantly improved the diagnosis of VL by providing accurate and rapid results outside traditional laboratory settings. These tests utilize immune responses to antigens or antibodies, allowing for efficient and simple diagnostics.[36] Recent advancements in immunochromatographic techniques have led to the development of specific monoclonal antibodies such as A6A2 and E3C3, which exhibit high sensitivity (95.8%) and specificity (98.7%) for detecting VL. The use of immunochromatographic assays has enabled the detection of VL in just 15 min, even in patients with weakened immune systems, including young children.[37] Rapid and accurate tests in a strip format with minimal instruction, making it suitable for use in remote and resource-limited settings have revolutionized point-of-care testing for VL diagnosis. These tests have undergone improvements to increase their durability and stability, enabling their use in diverse and challenging environments. Additionally, the enhanced formulation of RDTs allows for the detection of minute amounts of antigens or antibodies, leading to increased sensitivity and the ability to detect VL at an earlier stage of infection, thereby reducing false negatives.[38,39]

The rK39 test, in particular, detects antibodies against a recombinant antigen derived from the kinesin protein of *Leishmania*. It provides results within 10–30 min and requires minimal laboratory infrastructure, making it suitable for use in endemic regions. In a meta-analysis for VL diagnostics, among five index tests, rK39 ICT (sensitivity of 91.9% and specificity of 92.4%) has outreached KAtex LAT in urine (sensitivity of 63.6% and specificity of 92.9%), fast agglutination test, rK26 ICT, and rKE16 ICT.[30,40] However, the sensitivity varies significantly by geography; for instance, it is lower in East Africa (85.3%) than in the Indian subcontinent (97.0%).[39]

In antigen-based serological tests, recombinant antigens are favored over natural antigens due to issues associated with cross-reactivity and thus potential for false-positive results.[41] Lateral flow assay based on purified *L. donovani* promastigote membrane antigens from *L. donovani* promastigotes showed 96.49% sensitivity and 95% specificity with serum samples for diagnosing human VL in India. With urine samples, the sensitivity was 95.12%, and the specificity was 96.36%. For the diagnosis of Brazilian VL using patients' sera infected with *L. infantum*, the antigens exhibited a sensitivity of 88.57% and a specificity of 94.73%.[9,42]

Flow cytometry for serological *Leishmania* detection is a recent development. It can quantify antibodies rapidly with lower sample input volumes than other serological tests.[43]

Serological tests offer several advantages, including noninvasiveness and relatively high sensitivity and specificity; however, they also have limitations, such as cross-reactivity with other infections and the potential for false negatives in immune-compromised patients. They are also not suitable as prognostic assays due to the presence of antibodies from previous VL episodes. Therefore, serological testing is often employed in conjunction with clinical evaluation and other diagnostic methods, such as microscopy or polymerase chain reaction (PCR), to confirm the presence of the disease. Serological tests are not typically used in areas where CL is prevalent because of the low levels of circulating antibodies. Additionally, in regions with cross-reacting parasites like *Trypanosoma cruzi*, the specificity of these tests can vary.

Molecular diagnostics

Molecular diagnostics for VL have gained prominence due to their higher sensitivity and specificity than traditional diagnostic methods.[44] Molecular tests hold significant potential in the case of human immunodeficiency virus (HIV)-VL co-infection due to the decreased antibody

response observed in HIV-infected patients, which subsequently reduces the sensitivity of serological tests.[45] Molecular techniques, primarily based on PCR and its variants like nested PCR,[46] multiplex PCR,[47] oligochromatography–PCR,[48] and amplified fragment length polymorphism[49] enable the precise detection of *Leishmania* deoxyribose nucleic acid (DNA) in various biological specimens, such as blood, bone marrow, and skin lesions, thus facilitating early diagnosis and timely treatment. In VL, samples from the spleen, bone marrow, or lymph nodes are used for testing, with varying levels of sensitivity.[44] Peripheral blood testing using PCR can range in sensitivity from 62% to 93.2%, depending on factors such as the timing of sample collection during the infection process or the lower levels at which certain *Leishmania* species may circulate in the peripheral blood.[50] The slit skin smears and skin biopsies are frequently utilized for the detection of *Leishmania* DNA in PKDL; recently, the utility of peripheral blood was also explored for the diagnosis of PKDL.[51,52]

PCR can detect asymptomatic or presymptomatic infection, helping in control measures and blood donor screening.[53] Nested PCR improves upon conventional PCR by enhancing sensitivity and specificity, but it requires more time and carries a risk of cross-contamination during setup. A modified *Leishmania* spp.-specific nested PCR has demonstrated 100% sensitivity and specificity while reducing carryover and cross-contamination.[54]

Quantitative PCR techniques can assess parasitic load and provide valuable prognostic information. A study showed that the presence of 10 parasites/mL of blood after treatment indicated relapse, offering useful information for disease prognosis.[55,56] According to another study conducted by Verrest *et al.*[57] that used qPCR-based parasite quantification for treatment response, a cutoff of 20 parasites/mL at day 56 following the commencement of treatment was a very sensitive predictor of relapse within 6 months. A *Leishmania* kinetoplastid-targeted qPCR quantified parasite load up to 75 parasites/μg genomic DNA at the time of relapse posttreatment.[58] PCR performance depends on factors such as the nucleic acid extraction method, sample type, gene target copy number, and primer design.[59] Real-time PCR exhibits higher sensitivity and specificity (93.9% and 100%, respectively) was reported than in the PCR assays tested [75.6% and 100% for kinetoplast DNA (kDNA), and 53.7% and 88.8% for internal transcribed spacer region 1 (ITS1), respectively].[60] The bisulfite modification technique serves to simplify the genome before the implementation of PCR and has been geared for the detection of *Leishmania* in real-time PCR.[61]

Droplet digital PCR (dd-PCR) enabled the absolute quantitative measurement of target DNA, negating the need for calibration curves in PCR assays.[62] Expression analysis of amastigote-specific virulence genes, *A2* and *amastin* by dd-PCR in *L. donovani* had demonstrated an invaluable asset for drug development and monitoring disease progression.[63] A dd-PCR based on 18S rDNA was developed and validated for seven *Leishmania* species; however, despite its accurate quantification of DNA, the dd-PCR assay was found to be slightly less sensitive and specific than an equivalent real-time PCR assay (84.0% for dd-PCR vs. 85.0% for real-time PCR).[64] Additionally, the high cost of droplet digital PCR, which is three times that of real-time PCR, restricts its widespread use for routine diagnosis of leishmaniasis.

After real-time PCR, melt curve analysis is used to identify the specific *Leishmania species* by examining the melting profile (melting curve and melting points) of species-specific conserved sequences such as ITS1 and 7SL RNA genes.[65,66] This method's effectiveness depends on the fact that the temperature at which a sequence of double-stranded DNA dissociates, or "melts," is determined by the guanine–cytosine/adenine–thymine ratio and the length of an amplicon.[67]

The loop-mediated isothermal amplification (LAMP) method is a simple, rapid diagnostic tool for nucleic acid detection with high accuracy and robustness. LAMP involves amplification at a single temperature and a visible color change, which can be detected by the naked eye or under blue light, and, more recently, in real-time by fluorimetry.[52,68] LAMP has a reported sensitivity of 80%–98.3% and specificities of 94%–100% for human leishmaniasis diagnosis.[69,70]

Recombinase polymerase amplification (RPA) and recombinase-aided amplification (RAA) are used for isothermal amplification of kDNA minicircle to detect *Leishmania*. The sensitivities and specificities of RPA and RAA were 100% and 100%, and 65.5% and 100%, respectively.[71,72] In a recent study, RPA showed agreement with quantitative PCR-positive VL (96%) and PKDL (91%) cases, suggesting that it could develop into a field-applicable molecular tool for parasite load monitoring in resource-limited settings.[73] RPA combined with a rapid extraction method to detect *L. donovani* resulted in rapid, mobile detection systems that avoid the need for refrigerated reagents making it field applicable, however, both methods are time-consuming and labor-intensive.

The next-generation sequencing allows for comprehensive profiling of *Leishmania* species and their genetic variability,

aiding in the understanding of transmission cycles, detection of parasite variants with new clinical features, and identification of genetic markers related to drug resistance and virulence.[74] Whole genome sequencing was used to analyze parasites from clinical samples of VL patients due to *L. (L.) donovani*. Agilent SureSelect technology enabled target enrichment of *L. donovani* DNA, allowing direct genome analysis in clinical samples, and overcoming challenges of high human DNA levels and parasite load variation.[75,76]

Imaging techniques

Traditional diagnostic methods predominantly rely on serological tests, clinical evaluation, and bone marrow or splenic aspirates, but imaging modalities such as ultrasound,[77,78] computed tomography (CT),[79,80] and magnetic resonance imaging (MRI),[81,82] have increasingly become important adjuncts in the diagnostic process. Ultrasound is particularly valuable due to its ability to reveal hepatosplenomegaly—a hallmark of the disease—alongside other potential complications, such as lymphadenopathy and ascites, which are often seen in advanced cases.[83] CT scans provide detailed visualization of abdominal organs and allow assessment of granulomatous lesions, organ involvement, and complications like opportunistic infections or malignancies and help clinicians distinguish VL from other infectious conditions. MRI, though less commonly employed due to its accessibility and cost, can also be helpful in certain cases by providing exquisite detail of soft tissue characteristics and staging of the disease, particularly in rarer presentations involving the central nervous system.[84] Collectively, these advanced imaging techniques not only facilitate more accurate diagnosis but also assist in monitoring the response to treatment and the progression of the disease, underscoring the importance of integrating multimodal diagnostic approaches to improve patient outcomes in VL.

ROLE OF ARTIFICIAL INTELLIGENCE (AI) IN DIAGNOSTICS

Recent advancement in AI, through its two strong pillars-ML and deep learning (DL) algorithms, has exhibited enormous success in the field of medical diagnosis, treatment planning, patient monitoring, and personalized care in recent times. A subclass of DL algorithms, known as convolutional neural networks (CNN), has achieved tremendous success in image data classification, including those related to dermatology, radiology, and pathology. Various research underlines the results of dermatological diagnosis using CNNs, which proves that such models can achieve very high accuracy and even outperform human dermatologists in certain cases.[85-89] AI, in conjunction with advanced instrumentation and application methods, has upgraded the world of diagnostics. AI enhances diagnostic procedures in several key respects.

Image analysis

AI algorithms are also increasingly applied to medical imaging done for parasitic detection. While minute details in the photos might escape human observers, on the other hand, ML algorithms can be trained for the very same thing. AI-driven image analysis will speed up diagnosis by recognizing the shapes of parasites on tissue samples or blood smears with better precision.[90,91] Pierre *et al.* performed a new optimized way of diagnosis of CL, including automatic early skin lesion detection with an accuracy of 96%, specificity, and sensitivity of 94% and 92%, respectively.[92] DeepLeish, a DL system, was used to detect the Leishmania parasite from microscope images obtained from VL and CL patients. YOLOv5 showed the highest detection capability with a mean average precision of 73%, and precision and recall rates of 68% and 69%, respectively.[90] The model proposed by Ababaker Mohamed Noureldeen *et al.*[93] used the YOLOv5 algorithm for the detection and classification of CL infection from mobile phone images with an average accuracy of 70%, with sensitivity and specificity of 99% and 98%, respectively.

Predictive analytics

Many symptoms, epidemiological data, and the results of diagnostic testing through big data in AI models can estimate the probability of VL. Predictive analytics has great potential to support high-risk population identification with higher accuracy and enable appropriate action. Leal *et al.*[94] used the DL algorithm AlexNet to identify CL from 26 skin diseases with 2458 images with an accuracy of 95.04%.

Data integration

AI systems integrate data from various tests, including rapid tests, biomarker testing, observations in the clinical setting, and patient records, to provide a complete picture of the patient's health state. This type of integrated information, after analysis using an AI-powered platform, will provide faster diagnostic outcomes with increased accuracy.[91]

Limitations and challenges

VL presents several limitations and challenges in its diagnosis, which can significantly hinder its effective management and treatment. One of the primary challenges is the overlap of symptoms with other febrile illnesses, such as malaria and dengue, which can lead to misdiagnosis, especially in endemic regions where these diseases are prevalent. The traditional diagnostic methods, such as parasitological examination,

rely on invasive procedures like bone marrow aspiration or splenic puncture, which not only risk complications but also require skilled personnel and specialized laboratory facilities that may be lacking in resource-limited settings. Furthermore, serological tests, although less invasive, often suffer from issues of sensitivity and specificity, particularly in immunocompromised individuals or those with co-infections, yielding false negatives or positives.[45,95] Molecular techniques, including PCR, offer improved sensitivity and specificity but may not be readily accessible due to the need for sophisticated equipment and trained technicians, thus limiting their utility in many endemic areas. Another challenge in diagnostics is identifying drug-resistant strains, as the identification of resistant strains necessitates advanced genetic and molecular analyses that are not widely available. The lack of standardization in diagnostic tests, slow regulatory frameworks, and unequal distribution of diagnostic resources pose significant challenges to proper access to VL diagnostic tests in resource-limited settings. Overall, the multifaceted challenges inherent in the diagnosis of VL underscore the urgent need for the development and implementation of rapid, non-invasive, and reliable diagnostic tools that can be deployed in diverse healthcare settings to facilitate timely intervention and mitigate the morbidity and mortality associated with this devastating disease.

GLOBAL EFFORTS AND INITIATIVES

Recognizing the urgent need for effective diagnostics to manage and control this disease, various global funding initiatives have emerged in recent years aimed at enhancing the development and accessibility of VL diagnostic tools. Organizations such as the Bill & Melinda Gates Foundation, the Drugs for Neglected Diseases Initiative, and the WHO have played pivotal roles in mobilizing resources and fostering partnerships among governments, researchers, and nongovernmental organizations and supporting innovative diagnostic technologies for early VL diagnosis and treatment in low-resource settings and integrating these diagnostic tools into national health systems. International collaborations have fostered knowledge sharing and capacity building in endemic regions, enabling local health systems to better utilize these diagnostic tools. As these collaborative efforts continue to evolve, they hold the promise of significantly reducing the burden of VL and improving the quality of life for those at risk.

FUTURE DIRECTIONS/CONCLUSIONS

Recent advancements in molecular techniques have significantly improved the accuracy of identifying *Leishmania* DNA in patient samples. This has been particularly beneficial in endemic areas where traditional diagnostic methods may lack sensitivity and specificity due to potential co-infections.

Point-of-care tests are being developed, utilizing novel biomarker detection systems such as LAMP, which allows for rapid and reliable diagnosis in remote or resource-limited settings. Integrating AI and ML algorithms into diagnostic workflows shows promise in analyzing complex datasets and predicting disease outcomes, facilitating timely interventions. Furthermore, advancements in imaging technologies, such as high-resolution ultrasound and CT, provide noninvasive methods to assess visceral organ involvement in suspected VL cases, enhancing the overall diagnostic approach. Biomarker Panels can bring multiple biomarkers onto one diagnostic platform to enhance detection accuracy to provide a more comprehensive diagnosis for VL. Tests with biomarker panels will be most useful in the regions where VL is endemic along with other infectious diseases, helping in the differential diagnosis of VL from other diseases like malaria, tuberculosis, schistosomiasis, and lymphoma having similar clinical manifestations.

Integrating VL control into broader public health frameworks, can enhance resource allocation and improve health outcomes. Long-term success is pegged on being able to support countries in the development of sustainability plans that incorporate the prevention of vector-borne infection into the general health policies of nations (WHO).

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Conflicts of interest

There are no conflicts of interest.

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Review Article

Unraveling novel biomarkers in Indian post-kala-azar dermal leishmaniasis using proteomics

Anjali Singh, Susraba Chatterjee, Akrite Mishra, Sumi Mukhopadhyay

Department of Laboratory Medicine, School of Tropical Medicine, Kolkata, West Bengal, India

Abstract

Recent years have seen a rise in the use of mass spectrometry (MS)-based methods in proteome profiling research. MS-based proteomics has been increasingly implemented in various disciplines to identify and quantify biomolecules in a variety of biological specimens. MS-based proteomics is increasingly being applied for biomarker discovery due to its high sensitivity and specificity making it superior to any other available technology. Post-kala-azar dermal leishmaniasis diagnosis is challenging due to the low parasite burden and its symptomatic resemblance with other diseases like vitiligo or leprosy. Therefore, it is imperative to identify promising biomarkers with translational utility. The development of point-of-care assays using these biomarkers holds considerable potential for successful case diagnosis and for the efficient implementation of the ongoing Kala Azar Elimination Program.

Keywords: Mass spectrometry, PKDL, proteomics

Address for correspondence: Dr. Sumi Mukhopadhyay, Department of Laboratory Medicine, School of Tropical Medicine, Kolkata 700073, West Bengal, India.
E-mail: drsumimukhopadhyay@gmail.com
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INTRODUCTION

Leishmaniasis is a neglected tropical disease of poverty with a global public health impact and is caused by a protozoan parasite of the genus *Leishmania* and transmitted by the *Phlebotomine* sandfly. Leishmaniasis is widespread, currently placing a global population of approximately 1 billion at risk.[1] Leishmaniasis often flares up in areas of low endemicity into epidemic proportions due to natural or man-made disasters, including famine, drought, flood, earthquakes, and wars.[2]

Leishmaniasis is classified as a category I illness by the World Health Organization (WHO) as it is an emerging and uncontrolled disease, and the World Health Assembly has recognized it as a serious public health problem in its resolution 43.18.[3] The visceral leishmaniasis (VL) elimination framework, identifies early diagnosis along with effective case management, active disease and vector surveillance, social mobilization and building partnerships, and clinical and operational research as five key strategies for achieving the elimination program.[3] To fulfill the Sustainable Development Goals, WHO recently defined a road map for the prevention, control, and eradication of 17 neglected tropical diseases, including VL, by 2030.[3] At the sub-district or district level, the elimination program's goal was to bring the annual incidence of VL below 1/10,000 people. The VL Elimination Program has four phases; a pre-control "preparatory" phase, an "attack phase" to bring down the case incidence below 1/10,000 populations, a "consolidation phase" of 3 years to keep the incidence

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below the target, and a "maintenance phase" to ensure the maintenance of post-elimination status beyond 2020.[4] The initial elimination target year of 2015 was deferred to 2017, then to 2020, and 2023 at present.[4] Efforts to prevent infection or interrupt transmission include vector control strategies as well as evaluating the role of asymptomatic individuals and post-kala-azar dermal leishmaniasis (PKDL) cases. Because of the anthroponotic disease pattern, the parasite-loaded papulonodules in PKDL play a pivotal role in transmission and act as an important disease reservoir.[5]

Epidemiology of PKDL

PKDL is a well-recognized cutaneous sequela of kala-azar or VL. PKDL was first described by Dr U. N. Brahmachari in 1922 as a distinct clinical entity separate from other forms of cutaneous leishmaniasis.[6] *Leishmania donovani* a protozoa that causes VL was established as the causative pathogen and it was hypothesized that the cutaneous manifestations were due to antimonial therapy, which attenuated the pathogen so that it was restricted to the skin sparing the internal organs. Accordingly, he named the entity "dermal leishmanoid" in agreement with the fact that attenuated smallpox virus was called "vaccinoid." In the following years, many such cases were reported from the Calcutta School of Tropical Medicine and Madras Medical College.[6] In 1927 Acton and Napier coined the name "post-kala-azar dermal leishmaniasis" to differentiate it from oriental sore which was referred to as "dermal leishmaniasis."[6]

PKDL has considerable epidemiological importance as they are known to harbor the causative pathogen *L. donovani* in the dermal lesions and thus aid in the anthroponotic circulation during inter-epidemic periods. PKDL has been earmarked as one of the major obstacles to the successful implementation of the Kala Azar Elimination Program. PKDL is mainly reported from six countries, India, Sudan, South Sudan, Bangladesh, Ethiopia, and Nepal. In India, PKDL cases have been found in 54 districts, of which 33 are in Bihar, 11 in West Bengal, 4 in Jharkhand, and 6 in Uttar Pradesh. In West Bengal, mainly five districts have reported cases of PKDL, these include Darjeeling, Uttar Dinajpur, Dakshin Dinajpur, Malda, and Murshidabad.[7]

Indian PKDL typically presents in two distinct clinical forms, "polymorphic PKDL" and "macular PKDL,"[8] and are generally not self-healing. These dermal lesions usually manifest following an average period of 3.13 years and subsequently get spread to extremities. In the Indian context, between 1997 to 2019, studies determined that 15%–31% of PKDL patients in India had macular lesions, whereas 69%–85% exhibited polymorphic lesions.[9] However, due to the implementation of active case surveillance in West Bengal, a huge number of macular cases were discovered, and now macular PKDL constitutes nearly 50% of the PKDL population burden.[10]

Diagnostic approaches in PKDL

Unlike polymorphic PKDL, macular cases pose a diagnostic challenge, due to the negligible presence of demonstrable parasites, diagnosis is thus solely by clinical features, and their hypo-pigmented lesions are often indistinguishable from other hypopigmentary disorders like vitiligo, pityriasis versicolor, or leprosy.[10] Recent studies have highlighted that the macular PKDL was less responsive to liposomal amphotericin B,[10] suggesting that there might be possible differences in host–parasite interactions, further emphasizing the need for considering treatment stratification. To completely eliminate Kala-azar in Southeast Asia, proper identification of various routes responsible for the spread of the disease is essential,[11] which could efficiently distinguish between active PKDL patients and cured individuals as well as between active PKDL patients and another cross disease like leprosy and vitiligo. Most of the diagnostic methods for PKDL disease identification are based on highly invasive tissue biopsy methods followed by polymerase chain reaction (PCR)/quantitative PCR, which requires high technical expertise and is unsuitable in field settings. Salotra *et al*.[12] demonstrated a high sensitivity (95.45%) and specificity (93.5%) of rk39 ELISA for diagnosing PKDL subjects from Bihar. Furthermore, a study by Mathur *et al*.[13] showed that rk39 was 100% sensitive and specific for PKDL subjects enrolled at a tertiary care center in North India. Ejazi *et al*.[14] have demonstrated two urine-based immunoassays for diagnosis of PKDL patients, the sensitivity and specificity of urine-enzyme-linked immunosorbent assay (ELISA) were 97.94% (95/97) and 100% (75/75), respectively. Goswami *et al*.[15] also demonstrated the utility of testing urine samples with rK39 strip for the diagnosis of PKDL. However, all these studies had limited sample size to reach a conclusive result. A similar study by Singh *et al*.[16] also highlighted the diagnostic utility of rk39 ELISA. Verma *et al*.[17] demonstrated the utility of nested PCR, which had 91.1% sensitivity, whereas imprint smear microscopy had a sensitivity of 70.9% for the diagnosis of PKDL patients. Salotra *et al*.[18] demonstrated a Species-Specific PCR assay for the detection of *L. donovani* in clinical samples from patients with kala-azar and PKDL. The assay could successfully diagnose 45 of 48 PKDL cases (93.8%). Sreenivas *et al*.[19] also reported a nested PCR assay for the detection of *L. donovani* in slit aspirates from PKDL lesions. PCR results were positive in 27 of 29 (93%) samples by nested PCR assay, whereas only 20

of 29 (69%) were positive in a primary PCR assay. The nested PCR assay allowed reliable diagnosis of PKDL in a noninvasive manner. Verma *et al*.[20] have developed a rapid loop-mediated isothermal amplification assay for diagnosis and assessment of cure of *Leishmania* infection, with a sensitivity of 97%. A study conducted by Ganguly *et al*.[21] in an endemic area of Malda District, West Bengal, India, reported that a species-specific PCR on slit skin smear demonstrates a sensitivity of 93.8%. More recently, Moulik *et al*.[10] have reported the usefulness of internal transcribed spacer-1 PCR, and quantification by amplification of parasite kinetoplastid deoxyribonucleic acid in skin biopsies of PKDL patients enrolled from endemic districts of West Bengal. The research concluded that measuring the number of parasites is a useful method for keeping track of PKDL patients. It was found that miltefosine almost completely cleared the parasites and alleviated the symptoms. On the other hand, in instances where LAmB was used for treatment, the presence of parasites indicated that the treatment was not sufficient. Jaiswal *et al*.[22] reported the development of a new assay glycosylated circulating immune complexes with a sensitivity of 95.6%, specificity of 99.3%, negative predictive value of 97.1%, and positive predictive value of 98.9% for the diagnosis of PKDL patients from different endemic districts of West Bengal. This study also identified some glycoproteins whose expression was upregulated in PKDL compared with leprosy or vitiligo. Taken together, a study utilizing strip tests like rk39 strips on patients hailing from Bihar is highly sensitive and reliable for PKDL, however for macular PKDL sensitivity was only 73%. PCR-based methods of diagnosis are highly sensitive and specific, however, taking into consideration the rural nature of the disease, they are costly and not field-adaptable. Currently, there are several contemporary methods and instruments available for PKDL diagnosis. Parasite identification by skin slit smear is considered the gold standard but has a sensitivity of only 58% for PKDL case detection as highlighted by studies in Bihar.[23] In addition, the *Leishmania* skin test for determining parasite load or culture isolation from skin biopsies is also not reliable, as it has only 54% sensitivity. Histopathological studies with patients' skin biopsies too have very low (7%–33%) sensitivity towards macular case detection.[23] There are several analytical methods available for diagnosing both VL and PKDL, however, there are not many documented prognostic indicators or biomarkers to track how well VL/PKDL is responding to treatment. When distinguishing between an active VL/PKDL case and a cured VL/treated PKDL case, these indicators would be quite helpful. Since PKDL patients serve as silent parasitic reservoirs of *L. donovani* in VL-affected regions and actively participate in anthroponotic VL transmission, hence there exists an urgent need for the identification of novel biomarkers and the development of an accurate diagnostic and prognostic assay. In light of the increasing load of recurrent PKDL cases in endemic districts of West Bengal[24] the need for such biomarkers is now even more demanding.[24]

Role of proteomics in discerning novel biomarkers

Proteomics has revolutionized biomedical research in the post-genomic era. With the advent of the technological revolution and emerging computational and statistical programs, proteomic methodology has evolved rapidly in the past decade and shed light on solving complicated biomedical problems. Proteomics, enables us to analyze protein composition, structure, expression, modification status, and the molecular mechanism associated with diseases as well as aid in the discovery of novel biomarkers, which could be utilized in specific diagnostic assays, prognostic predictors, and therapeutic targets to enhance personalized medicine.[25,26]

However, due to variations in protein expression based on time and environmental factors, proteomics is significantly more complex than genomics. Proteomics methods are diverse and include two-dimensional and one-dimensional gel electrophoresis.[27] Disease markers are extensively used for screening, diagnosis, staging, prognosis, monitoring response to treatment, and recognition of repetitive diseases.[28] It is also necessary for mapping the intricately linked networks, pathways, and molecular systems that directly regulate the main processes of life, including apoptosis, senescence, differentiation, and proliferation of cells. Over the past 10 years, experimental technology has significantly improved. Advancements in proteomic methodologies over conventional methods, such as immunohistochemistry staining, western blot, and ELISA to high-throughput methods such as tissue microarray, protein pathway array, and mass spectrometry (MS) have a substantial influence on biomarker research[29] [Figure 1]. One frequently used method for proteome analysis is MS. This protein identification technique, which is typically used in conjunction with a liquid chromatography system, uses fragment detection and measurement. Peptide sequences present in samples can be accurately identified by comparing the results to large-scale databases.[30] By comparing protein changes between healthy and diseased conditions, an MS-based proteome-wide analysis can be used to identify pathogenic mechanisms at the phenotypic level. Furthermore, it is possible to quantify a particular stimulus, which can include anything from variations in the concentration of a single protein or its identified post-translational modification (PTM) to the proteome-wide

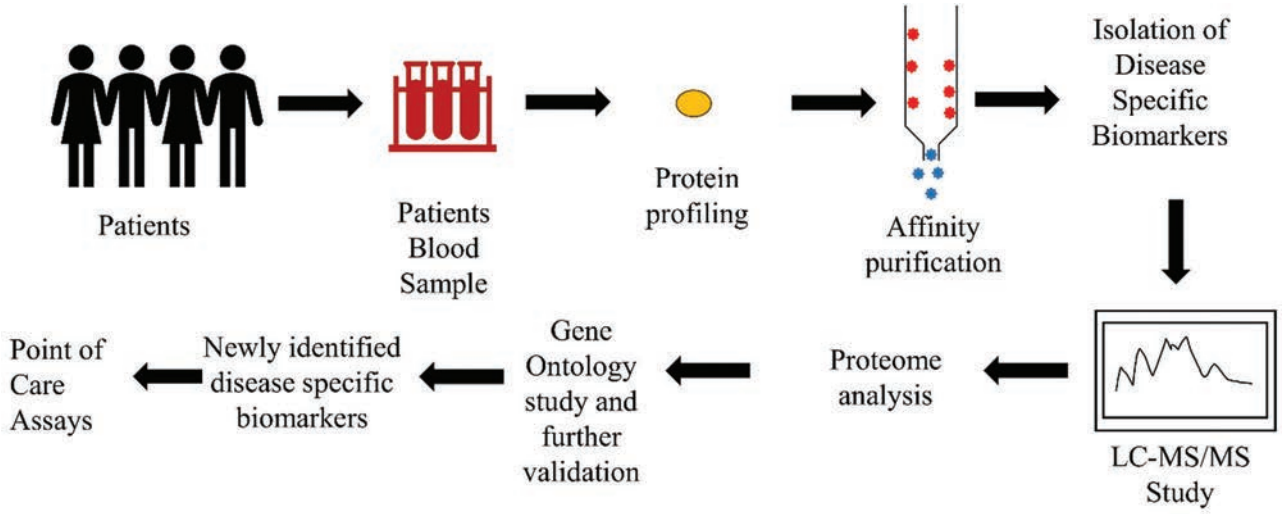

Figure 1: It represents a schematic diagram of mass spectrometry-based proteomics analysis in post-kala-azar dermal leishmaniasis samples to identify biomarkers for the development of point-of-care assays

kinetics of the same modification at various phases of the cell cycle. More precise and trustworthy protein and PTM quantification is essential for developing novel disease-related biomarkers for improved diagnosis, prognosis, and therapy.[31] Hypothesis-free MS-based proteomics offers a major benefit in that it does not require making any assumptions about the potential nature and number of biomarkers, which is a significant departure from traditional biomarker research that focuses on single protein measurements. In essence, MS-based proteomics encompasses all hypothesis-driven biomarker studies for each disease and also establishes the connection between potential biomarkers. The identification of additional biomarkers using proteomics approaches is likely to be successful, especially for certain diseases such as cancer, human immunodeficiency virus, tuberculosis, cardiovascular diseases, and many more. It is important to recognize that most currently used biomarkers are either found in high quantities or are linked to known physiological conditions. When we consider the ratio of biomarkers to proteins in the high abundance range and extrapolate it to lower abundance proteins, it suggests the possibility of discovering several hundred new biomarkers using suitable technology.[32] In the field of proteomics, attempts have been undertaken to identify and classify plasma proteins due to their potential use as disease biomarkers, for therapeutic monitoring, and in comprehending host responses to pathogens. Nevertheless, there are significant obstacles when utilizing plasma for proteomic/glycoproteomic analysis, including the wide range of abundant proteins, varied heterogeneity, the concealment effects of highly abundant proteins, and the extremely low abundance of certain crucial proteins. Consequently, the removal of highly abundant proteins is crucial to enhance the visibility of other important proteins.[33]

Blood-based diagnostics are ubiquitous in medicine, with biomarkers playing a vital role in patient categorization and treatment decision-making. However, routine blood biomarkers have significant limitations as they lack comprehensiveness and have limited sensitivity and specificity.[33]

Blood plays a vital role in human physiology and its composition reflects an individual's state or phenotype. The plasma proteome in particular offers valuable insights into overall health. Plasma proteome can be categorized into three broad classes. The first contains abundant proteins which have a functional role in blood. These include human serum albumin, apolipoproteins, acute-phase proteins, and proteins of the coagulation cascade. The second class includes tissue leakage proteins like aspartate aminotransferase and alanine aminotransferase, which are used for the diagnosis of liver diseases. The third class includes signaling molecules like small protein hormones and cytokines, which typically have very low abundances in a stable state. This complex plasma proteome landscape underscores the challenges and opportunities in biomarker discovery and clinical diagnostics.[33]

According to Food and Drug Administration (FDA)-National Institutes of Health: Biomarker-Working-Group, 2016 a biomarker is a defined characteristic that is, measured as an indicator of normal biological processes, pathogenic processes, or a response to an exposure or intervention. To date, there are more than 100 FDA-approved clinical plasma or serum tests, mainly in the abundant, functional

class (50%), followed by tissue leakage markers (25%), and the rest include receptor ligands, immunoglobulins, and aberrant secretions. MS-based proteomics has been a game changer in protein analysis, compared with traditional enzymatic and antibody-based methods, MS-based proteomics offers unparalleled specificity and unbiased analysis.

Novel biomarker of PKDL

To date, very few studies on leishmaniasis have reported altered plasma proteome of VL patients compared with healthy subjects. These studies reported various acute-phase proteins to be expressed differentially in VL patients compared with healthy subjects. Up-regulation of α-1-acid glycoprotein, C1 inhibitor, α-1-antitrypsin, α-1-B glycoprotein, and amyloid-A1 precursor; and downregulation of retinol-binding protein and vitamin-D binding protein were reported. These studies highlight the role of proteomics in identifying disease-specific protein markers that can help in the early detection of VL when parasite load is scanty.[34]

Jaiswal *et al.*[35] have characterized the detailed proteome profile of circulating immune complexes of Indian PKDL patients. The study identified 32 up-regulated and 11 down-regulated proteins among macular Indian PKDL patients compared with healthy subjects. Similarly, 18 up-regulated and 42 down-regulated proteins were observed among macular PKDL patients compared with polymorphic PKDL patients. Moreover, 71 up-regulated and 19 down-regulated proteins were observed among macular PKDL patients compared with cured individuals. These proteins were largely linked to inflammatory response, immune system process, and transport, which could be following various dermal manifestations linked with PKDL patients suffering from macular and polymorphic lesions.

For the first time, based on the proteomics study supported by western blot and ELISA, higher levels of circulating glycoproteins; plasminogen, vitronectin, and plasma cytokine transforming growth factor beta, among PKDL patients with macular and polymorphic lesions, compared with healthy and cured, additionally it was demonstrated to serve as PKDL disease severity marker.[34] Interestingly both plasminogen and vitronectin have been demonstrated to interact with *L. donovani* promastigotes suggesting their important pathophysiological role in Indian PKDL.[35,36]

This study also revealed that the ratio of vitronectin versus plasminogen could serve as an efficient biomarker for the efficient diagnosis of PKDL patients suffering from *Mycobacterium avium* complex lesions.

Leishmaniasis in humans, presents a considerable challenge, especially in tropical areas. In the past 30 years, CL has become prevalent in Sri Lanka as a parasitic infection attributed to *L. donovani* zymodeme MON-37. Manamperi *et al.*[37] has recently reported proteome profiling of skin lesions of patients suffering from CL. The proteomic-based study unraveled a total of 1290 proteins, among these 69 proteins were found to be differentially expressed when compared with healthy controls. Among these proteins, 64 proteins were found to be up-regulated, whereas three proteins were down-regulated.[37] Interestingly, the study reported the presence of several pathways linked with viral infections such as viral gene expression as well as assembly of viral components at the budding site, indicating the chances of an endobiont virus along with *L. donovani* in the skin lesion. Apart from this, the study demonstrated the significant upregulation of IRE1, PERK, and ATF6, three transmembrane proteins, which play an important role in the protein folding capabilities of endoplasmic reticulum.[37]

CONCLUSION

Such type of in-depth studies with Indian PKDL skin lesions could aid in deciphering a plethora of information and unraveling novel pathophysiological markers, which eventually will be beneficial in the development and optimization of therapeutic and diagnostic assays.

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Conflict of interests

There is no conflict of interest.

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Review Article

Leishmaniasis in Sri Lanka: Surmounting obstacles toward achieving elimination as a public health problem by 2028

Shalindra Ranasinghe, Deepika Fernando[1], Nayana Gunathilaka[2], Kanchana Mallawaarachchi[3], Rajitha Wickremasinghe[4]

Department of Parasitology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda, Sri Lanka, [1]Department of Parasitology, Faculty of Medicine, University of Colombo, Sri Lanka, [2]Department of Parasitology, Faculty of Medicine, University of Kelaniya, Sri Lanka, [3]District General Hospital, Hambantota, Sri Lanka, [4]Department of Public Health, Faculty of Medicine, University of Kelaniya, Sri Lanka

Abstract

In the 1990s, Sri Lanka started reporting cases of cutaneous leishmaniasis (CL), which gradually increased to the current case incidence rate of over 3000/year. The causative strain of CL is *Leishmania donovani* MON-37, which is genetically different from the visceral leishmaniasis (VL)-causing strain in Sri Lanka. Visceral and mucosal forms are rare in Sri Lanka. The potential vector is *Phlebotomus argentipes*. Due to increasing CL case numbers, the Anti-Malaria Campaign was identified as the focal point in 2022 by the Ministry of Health (MoH) to control leishmaniasis and a WHO-funded situational analysis and the first National Strategic Plan (NSP) for prevention and control of leishmaniasis in Sri Lanka 2024–2028 were developed. During the situational analysis, a comprehensive literature review, meeting the stakeholders, visiting CL endemic areas and hospitals, and a SWOT analysis were carried out. The goal of the NSP is "To control cutaneous leishmaniasis for possible elimination as a public health problem in the future and prevention of VL and MCL." The two objectives are as follows: to reduce the annual incidence of CL < 5 per 10,000 population by 2028 (approximately 6600 cases) and to ensure zero mortality due to VL. The NSP had three strategic plans and five supporting areas. Each activity was well-described, and timelines were given to complete each task. This review describes the activities carried out by the MoH, the research work conducted so far, and the key points in the NSP recommended to eliminate leishmaniasis as a public health problem from Sri Lanka by 2028.

Keywords: Elimination, history, leishmaniasis, public health problem, Sri Lanka

Address for correspondence: Prof. Shalindra Ranasinghe, Department of Parasitology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Gangodawila, Nugegoda 10250, Sri Lanka.
E-mail: ishalindra@sjp.ac.lk
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INTRODUCTION

Sri Lanka is a tropical island with a land area of 65,610 km^2, situated between 5° 55′ and 9° 51′ north latitudes and between 79° 42′ and 81° 53′ east longitudes, 32 km to the south of the main land of India. The central parts of the country contain mountains. Most rainfall is received from both northeast and southwest monsoons, but inter-monsoonal rains, depressions, and convectional evening showers also contribute to rainfall. The mean annual rainfall ranges from 900 mm in the driest parts to 5000 mm in the

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wettest parts. The mean annual temperature varies from 27°C in the coastal lowlands to 16°C to the central highlands.[1]

Sri Lanka has nine provinces and 26 administrative districts. It has a population of ~ 22 million,[2] an average life expectancy of 74.2 years for males and 80.1 years for females (as of 2021),[3] a crude birth rate of 13.8 and a crude death rate of 6.0 per 1000 population (as of 2020),[4] and has a GDP per capita of US $ 3340 (as of 2022) and a − 7.8 real GDP growth (as of 2022).[2] In 2022, the country faced an unprecedented economic crisis, but has achieved some signs of recovery by now.[5]

Historically, there has been a close relationship both geographically and culturally between Sri Lanka and India, with eternal travel taking place between the two countries. In spite of having a close relationship with the Indian mainland, Sri Lanka has eliminated filariasis as a public health problem in 2015 and eliminated malaria in 2012 and has maintained zero indigenous cases of malaria since 2013.[6,7] However, among vector-borne diseases, dengue and cutaneous leishmaniasis (CL) exist as major public health problems in the country at present.[8]

HEALTH SYSTEM IN THE COUNTRY

Sri Lankan healthcare facilities comprise both public and private sectors. The public sector provides nearly 95% of inpatient care and around 50% of outpatient care. The Ministry of Health (MoH) is responsible for stewardship functions, policy formulation and health legislation, program monitoring and technical oversight, and management of health technologies and human resources in the tertiary care and other selected hospitals. The Provincial Ministries are responsible for the functioning of secondary and primary care institutions. By the year 2022, there were a total of 1500 healthcare institutes which contained 588 hospitals. There are 517 primary care institutes and 335 Medical Officer of Health offices within the country.[9]

LEISHMANIASIS

Leishmaniasis is a vector-borne neglected tropical disease complex caused by the parasite belonging to the genus *Leishmania*. The disease presents in three main clinical forms: visceral leishmaniasis (VL), the most severe form; muco-cutaneous leishmaniasis (MCL), the most mutilating form; and CL, the most common form that is typically confined to the skin and heals with a disfiguring scar. The disease is transmitted by the bite of an infected female sandfly belonging to the subfamily Phlebotominae.[10]

HISTORY OF LEISHMANIASIS IN SRI LANKA

VL

Until the recent past, it was believed that leishmaniasis is a newly emerged and established disease in Sri Lanka.[11,12] However, a recent review into Sri Lankan archives[13] revealed that leishmaniasis had been reported in Sri Lanka since 1904, the same era when the Leishmania parasite was identified by Sir William Leishman and Sri Charles Donovan and when post kala-azar dermal leishmaniasis (PKDL) and treatment for kala-azar was described by Sir U. N. Brahmachari.[14]

Case reports of VL in Sri Lanka are scarce. According to archived reports, in 1904, Dr. Aldo Castellani, a pathologist and a microbiologist and the Director of the De Soyza Bacteriological Institute of then Ceylon (re-named as the Medical Research Institute (MRI) of Sri Lanka in 1946-)[15] had described a laboratory-confirmed VL case, detecting Leishman–Donovan bodies in splenic smears stained with Romanowsky's stain in a 20-year-old diseased male, who had symptoms of pneumonia and hepatosplenomegaly.[13,16] Altogether, there had been 75 archived records in 1900, from 1910 to 1916 and from 1936 to 1938, until 1947 mentioning the term "kala-azar." Furthermore, the same authors[13] reported that in 1947, a report published by the MRI of Sri Lanka had mentioned that 21 out of 21,772 specimens were positive for kala-azar when subjected to the formol–gel test. There had been no other positive cases of kala-azar reported thereafter.[13] After these reports, the next confirmed case of VL was reported in 1973, in a British girl who had a short transit in Colombo. The location where VL was contracted was inconclusive according to this case report.[17] After a long lapse, a recent confirmed endogenous case of VL was reported in 2007[12,18] in a 36-year-old woman from the north–central province of Sri Lanka who had never been abroad. Both liver and bone marrow biopsies and rK39 rapid diagnostic test (rK39 RDT) had yielded positive results in this patient, and she had complete cure with intravenous sodium stibogluconate (SSG) 20 mg/kg/day for 28 days with no relapse or PKDL in a follow- up of 9 years.[18] In 2011, another endogenous VL case was reported from the Northern Province[19] in a 57-year-old man. A few more confirmed and suspected VL cases have also been reported.[17] However, there had been no other VL cases reported in the literature since 2017. This shows that although there had been *ad hoc* cases of endogenous VL, it has not become a public health problem in the country to date.

CL

In the historic reports (Administration Report of The Director of Medical and Sanitary Services), CL had been first mentioned in 1928.[13] From 1928 until 1938, a total of 33,414 CL cases were reported in the archived records and had strangely reported 165 deaths among them, leaving inconclusive evidence of the type of the disease and their diagnosis.[13] Since the last possible case of CL that was reported in 1938, it was only in the year 1992 that the first recent confirmed endogenous case of CL was reported from a teacher in the Southern Province of Sri Lanka.[11]

Impact of malaria control on endogenous leishmaniasis

The Anti Malaria Campaign (AMC) in Sri Lanka was established in 1911, and dichloro-diphenyl-trichloroethane (DDT) had been introduced in 1945 for control of the malaria vector. In 1958, an island-wide malaria eradication program was initiated by the government, in keeping with the WHO recommendations.[7] Current CL-endemic areas overlap with previous malaria-endemic dry and intermediate zones of Sri Lanka.[8] Therefore, it could be hypothesized that the absence of leishmaniasis records in the National Archives may be due to the sandfly vector control that took place as a collateral effect of indoor residual spay (IRS) that took place for malaria control. Furthermore, in 1999, Roll Back Malaria Partnership Initiative commenced. As we report zero indigenous malaria case since 2012 and remain in the "prevention of re-establishment of malaria phase" the AMC started practicing only reactive and proactive targeted vector control measures while reporting of imported malaria cases with targeted IRS and long-lasting insecticidal net usage.[20] It is worth noting that the exponential rise of CL cases seen from 2008 onward[8] may be due to the change in vector control measures conducted by the AMC in those pre-malaria areas.

Recent clinical presentations of leishmaniasis

Sri Lanka mostly reports CL. A wide variety of clinical presentations in CL have been reported ranging from papules, nodules, and ulcers to plaques. These lesions are typically not itchy and not painful.[21,22] We have also reported atypical variants of CL.[23-25] Mucosal involvement has also been reported infrequently.[26] In many demographic studies on CL, it has been revealed that more males are infected than females and most are young outdoor working adults, lesions are commonly single, located in the exposed areas of the body, and they are mostly less than 2 cm in diameter. However, large atypical lesions are also reported. The common type of lesions seen here are ulcerated and non-ulcerated lesions, which usually last for > 3 months by the time they seek treatment.[21,27]

Spread of CL and the current burden

Since 1992 until the year 2000, few sporadic cases of CL were reported mainly from the soldiers who were engaged in the civil war that took place in the north and east of Sri Lanka.[28] Due to the rising number of CL cases since 2000, the Ministry of Health Sri Lanka, declared leishmaniasis as a notifiable disease in 2008 with a special circular.[29] As the CL case numbers were seen to be gradually increasing since 2000, more civilians, especially the community involved in farming and other outdoor activities, and school children contracted the disease, indicating possible establishment of peri-domestic and outdoor transmission.[27] Similarly, CL was initially seen mostly in two provinces: north–central and southern, and a gradual spillover of the disease with temporal expansion was observed to adjacent geographic regions over the past 3 decades, and it is now reported from 25 out of the 26 districts.[8,22] Currently, more than 90% of CL cases are however reported in five districts: Hambantota, Anuradhapura, Matara, Polonnaruwa, and Kurunegala districts. Alarmingly, the Hambantota district reported a case incidence rate of 117.2 cases/100,000 population in 2018, and by 2018, there were seven more districts reporting > 10 new CL cases/100,000 population.[22,30,31] New foci are also reported from previously non-endemic areas.[32] Furthermore, since 2018, more than 3000 CL cases have been reported annually, which is a three-fold rise compared to annual case numbers reported from 2010 to 1017.[8] The cumulative case number from 2009 to December 2023 is close to 30,000.[8] It is also worth noting that these case numbers are passive case detection values as there is no proper active case detection program taking place in the country to date and that these values therefore indicate the tip of an iceberg.

Etiological leishmania parasite in Sri Lanka

Causative agents for both CL and VL were identified predominantly as *Leishmania donovani* zymodeme Mon-37.[33-35] It was proven in several whole-genome sequencing and animal studies, as well as CL case follow-up studies, that the CL-causing strain in Sri Lanka is naturally attenuated and essentially dermatrophic and that the CL- and VL-causing *L. donovani* zymodeme Mon-37 strains are genetically different.[36-38] However, visceralization of CL in two HIV-infected patients was recently reported. One of the patients succumbed by the time VL was diagnosed, and the second patient was lost to follow-up after HIV infection was revealed to him (personal communication with two consultant microbiologists). However, none of those cases were reported as case reports, which is a limitation of the Sri Lankan notification system.

HISTOPATHOLOGICAL CHANGES SEEN IN SRI LANKAN CUTANEOUS LEISHMANIASIS (SL-CL) WOUNDS

Studies on histology of SL-CL is limited. A study conducted in 46 histopathologically confirmed CL samples (with positive amastigotes) containing one papule, 21 nodules, five plaques, and 19 ulcers, observed the presence of hyperkeratosis in 91.3%, irregular acanthosis in 54.3%, parakeratosis in 34.8%, follicular plugging in 21.7%, and hyperplasia in 10.9% of the tested lesions.[39] Those authors reported the presence of marked inflammatory cell infiltrates in the dermis (composed of histiocytes, plasma cells, and lymphocytes), organization ranging from diffuse inflammatory infiltrates with parasitized macrophages to varying degrees of granuloma formation, and formation of ill-formed histiocytic to epithelioid granulomata. However, they had not detected prominent necrosis in the SL-CL lesions.[17] Their findings were similar with previously described *L. major* and *L. tropica* findings.[40,41] Another study investigating 50 skin biopsy samples revealed hyperkeratosis in 90%, acanthosis in 44%, and epidermal atrophy in 34%.[42] Both studies showed that macrophage activation plays a major role in control of the parasite within the lesion. Ill-formed coalescent granulomata (OR = 14.83) and diffuse dense dermal plasma cell infiltrate (OR = 74.25) also are significantly associated with SL-CL when compared to other granulomatous dermatitis.[43] However, more studies with a larger number of samples will provide more useful evidence on tissue impact caused by SL-CL and on wound healing.

Immunological evidence

Immunological milieu in relation to the lesion

Few studies have been conducted on the immunological milieu in SL-CL. In one study, an *in situ* immunopathological response was investigated using gene expression.[39] It was found that there was a significant upregulation of IFN-γ ($P < 0.001$) and downregulation of IL-4 ($P < 0.001$) gene expression in the CL lesions compared to controls (biopsy samples collected from non-CL minor surgical wounds). There was no statistically significant difference in IL-10, IFN-γ, and TNF-α gene expression levels between the CL and the control groups. Furthermore, the same authors detected a significant increase in the expression of both IFN-γ ($P = 0.018$) and TNF-α ($P < 0.001$) genes in lesions lasting for > 6 months compared to lesions lasting for < 6 months.[39]

There is another study that had investigated the expression of immune checkpoint inhibitors in response to SSG treatment of SL-CL lesions. Here, the authors detected reduced expression of programmed death-ligand 1 (PD-L1) and indoleamine 2,3-dioxygenase 1 (IDO1) proteins in the lesions when treated with intra-lesional SSG (IL-SSG) for 4 weeks compared to before treatment (baseline). Therefore, the authors proposed that PD-L1 expression can be used as a predictor of response to IL-SSG in SL-CL lesions.[44]

Serological evidence

Some studies had reported sero-prevalence (positive IgG) in some patients with SL-CL with absence of symptoms of visceralization both at the point of study and with follow-up of several months.[45] These positive antibody results were mostly detected with in-house enzyme-linked immunosorbent assays (ELISAs) and rapid diagnostic tests (RDTs), where local parasitic antigens were used. Also, few studies report some CL patients showing positive results when tested with commercially available rK39 RDT kits (16%).[46] Serological data available on SL-CL are variable and need further investigation. However, serology is not used in Sri Lanka to diagnose CL. Whether Sri Lankan CL induces systemic immunogenicity in some individuals is worth investigating.

MICROBIOME AND BIOFILM IN SL-CL WOUNDS

There is only one study that had investigated the microbiome and biofilm of SL-CL wounds so far. This study investigated 39 confirmed CL wounds, which showed that both wound swabs and biopsies taken from SL-CL wounds had significantly distinct microbiome profiles and lower diversity compared to unaffected skin and that 61% of SL-CL lesions had biofilms. However, this study does not describe the correlation between wound healing and presence of biofilms as most of the patients were lost to follow-up during the COVID-19 pandemic period.[47] It will be important to investigate the role played by the wound microbiome and biofilm in the healing process of SL-CL wounds so that intervention to promote early wound healing could be implemented.

Diagnosis

a) **CL**

The most widely used routine diagnostic technique for SL-CL is the Giemsa-stained slit skin smear,[48] preceded by histology, which are both available in some of the government hospitals where a dermatologist, a histopathologist, and a medical laboratory technician (MLT) or a public health laboratory technician (PHLT) are available. In addition to those, few validated PCR methods and *in vitro* culture are available only in some universities,

where they perform investigations for research studies. There are a few studies that have investigated the sensitivity and specificity of loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification assay (RPA), and florescent *in situ* hybridization.[49-51] However, more studies are needed for recommendations to be made to use these tests as standard investigations at the national level. Few real-time PCR methods with high sensitivity and specificity have also been developed for SL-CL.[52]

b) **VL**

The few VL cases reported were mainly diagnosed with bone marrow biopsy and histology and in a few instances aided with a positive rK39-RDT, culture, and PCR.[12,18,19] rK39-RDT is not available in the National Healthcare System so far. Asymptomatic VL was also reported with positive ELISAs, DAT, culture, and PCR in a few studies.[18,53] The available literature on diagnosis of Sri Lankan VL is very limited, most probably due to low case incidence.

Treatment

a) **CL**

First and only treatment guidelines for leishmaniasis were developed in 2013 by the Sri Lanka College of Dermatologists[54] which needs a timely update. Adhering to those guidelines, the first line of treatment for SL-CL is intra-lesional sodium stibogluconate (IL-SSG), if the lesions are small in size (< 3–4 cm in diameter), less than 5 in number, and are not located over a cartilage, joint, nose, or in periorbital areas. IL-SSG is administered until the lesion blanches (~ 1 mL/1 cm^2/week; containing SSG 330 mg BP/mL) until the lesion completely heal with complete re-epithelialization.[55] In those lesions where IL-SSG cannot be infiltrated, intra-muscular SSG is recommended. SSG has been included in the Ministry of Health Essential and Approved drug list annually.[56] However, SSG runs stockouts frequently.[57] There are two studies on radio frequency heat therapy (RFHT) reporting equally good response compared to IL-SSG in treating small lesions, but leading to more severe scarring due to 2nd-degree burns and blistering and rupture of blisters within the first week after RFHT treatment.[58] Meglumine antimonate, miltefosine, or pentamidine are neither registered nor available in the national health system so far.[56,57]

Non-responsiveness in CL to intra-lesional SSG

Non-responsiveness to IL-SSG was first reported in SL-CL in 2016.[23] Afterwards, development of treatment failure and delay in healing with IL-SSG had been reported in a cohort of 201 laboratory-confirmed CL cases which had shown 75.1% of treatment failure to IL-SSG.[55] However, there is no solid evidence yet to say that SL-CL has developed true resistance to SSG or not.

b) **VL**

Two cases of VL were successfully treated solely with intravenous (IV) SSG 20 mg/kg/day × 28 days.[18,19] Another patient was treated with liposomal amphotericin B (brand Fungizone) 3 mg/kg/day for 14 days in three cycles, and another was treated with IV-SSG 800 mg, daily for 26 days, followed by miltefosine 100 mg daily for another 24 days.[17] None of them develop recurrence or PKDL in the long-term follow-up.[17] Amphotericin B deoxycholate is a registered drug in the Ministry of Health Sri Lanka. However, AmBisome is neither registered nor available in Sri Lanka. Miltefosine is also neither registered nor available in Sri Lanka.

Vector in Sri Lanka

The potential sandfly vector *Phlebotomous argentipes* Annandale and Brunetti 1908 has been reported in Sri Lanka from as early as 1949.[59] There are three morphospecies of *P. argentipes* identified in Sri Lanka; *P. argentipes*, *P. anandale,* and *P. glaucus*.[60] *P. argentipes is* the predominant species reported in the country, and in addition, *P. stantoni* and *P. salehi* have also been reported. There are 16 *Sergentomiya* species reported in Sri Lanka.[61,62] *P. argentipes* have been reported in almost all leishmaniasis patients' peri-domestic environment, and Leishmania parasite DNA was detected in *P. argentipes* by molecular techniques.[17,63-65] So far, no other sandfly genus or species have been shown to harbor *L. donovani* DNA other than *P. argentipes* in Sri Lanka, demarcating *P. argentipes* as the putative vector. Unfortunately, there are still no studies that have demonstrated the full developmental cycle of the Leishmania parasite within *P. argentipes* to confirm it as the vector of leishmaniasis in Sri Lanka.

Climate change, CL case numbers, and sandfly density

There are limited number of studies comparing monsoonal rain patterns, temperature, windspeed etc., with CL case numbers. The available studies have shown to report different correlations between CL case numbers and climatic changes in different geographic areas of the country.[31] This may be due to delay in treatment-seeking behavior, prolonged incubation period of SL-CL, chronic nature of the disease, and variable climatic changes observed in different parts of the country. It will also be important to assess the sandfly density and its correlation with climate change, soil temperature, evaporation rate, etc.[66] One study reported that the sandfly density increases during a monsoonal period (October–February)

in the Anuradhapura district,[65] and the study conducted by another group[31] reported an increase in CL case numbers from March to April in the same geographic location. Comparing these two studies, it could be hypothesized that 1–2 months following the increase in the sandfly density and going through the incubation period (1–2 months), the CL case incidence could have increased by Mach–April following monsoon season. Therefore, it will be important to study the climate change, sandfly density, and CL case incidence patterns holistically to arrive at control measures in each geographic area where a high incidence of CL cases is reported. This will enable predictive modeling for epidemics and to initiate appropriate preventive measures.[67]

Insecticide susceptibility studies

Only two studies have been conducted in Sri Lanka to date on insecticide susceptibility on sandflies. One study had revealed elevated esterases and acetylcholinesterase as potential resistance mechanisms to insecticides in a catchment of *P. argentipes* in Northern Sri Lanka.[68] A more extensive recent study collecting sandflies from several CL-endemic areas of the country described that most sandflies are susceptible to deltamethrin and propoxur, except for malathion and DDT, which were used extensively in the country during malaria control.[69]

Reservoir host

Data available on the animal reservoir are inconclusive so far. There are only two studies investigating the animal reservoir, one reporting the presence of amastigotes in a Giemsa-stained slit skin smear and in only one of the blood smears in 151 screened dogs in a leishmaniasis-endemic area[70] and another study reporting one positive rK39-RDT in 114 screened dogs.[71] There are no further studies reported on animal surveillance for over a decade, and it has been recommended that active surveillance into animal reservoirs adhering to one-health approach be carried out. This is an important aspect in the control and elimination of leishmaniasis in Sri Lanka as well as from the South Asian region.[72]

Awareness

Social awareness and awareness among the health staff are supposed to be important in the prevention and control of both communicable and non-communicable diseases. In vector-borne diseases, not only about the disease, but awareness about the vector bionomics is also important to prevent man–vector contact and to interrupt transmission.[73] One recent island-wide study conducted among 252 confirmed CL cases and 2608 controls from the same geographic regions revealed that 79.1% interviewed knew that a fly induces CL, but the knowledge on vector breeding places, biting times, and preventive methods was poor. Knowledge on signs and symptoms of CL in this study sample had been good, and the awareness had been mainly obtained from the healthcare system and rarely from the mass media.[73] In contrast, another study reported lack of knowledge on early signs and symptoms of CL and therefore delayed health-seeking behavior in a different study cohort, and here the CL patients had commented that "we do not rush to hospital for ordinary wounds."[74] A third study in a different geographic region showed that only 28.9% of the study population knew about the vector and control measures.[75] This reveals that there is a dire need to improve social awareness among the community on vector control measures, early signs and symptoms of CL, and therefore motivation toward early health-seeking behavior to interrupt transmission and prevent disfigurement and also to ensure engagement of the community on vector control strategies.

Community engagement

Community engagement and use of appropriate interventions have proven to play a significant role in the control of communicable diseases.[76] There is only one community engagement study in CL in Sri Lanka reported to date; a multi-center study (ECLPISE), in which Sri Lanka was also a focal point. This study had a decolonial approach and had recruited community members, religious leaders, traditional healers, people with CL and their families, teachers, local administrators, and representatives of key community groups. They found out that the community involvement in Sri Lanka is culturally tailored and recommended that a better relationship between researchers and community members is vital to prevent mistrust and prevent the researcher from becoming an "uninvited guest" and make the community "doing engagement"[77] in the prevention and control of leishmaniasis.

Economic impact of CL

The economic cost had been evaluated in one study conducted in one of the CL-endemic areas in the country. This study reported that significant economic burden is caused due to CL. The total median economic loss to a household was calculated as 61.27 USD (Rs. 9927), and from the healthcare provider's perspective, the total median cost per patient was calculated as 22.83 USD (Rs. 3696).[78] Wider calculation of healthcare economics is also a dire need to the country, which has not been evaluated by the Ministry of Health so far.

Measures taken after leishmaniasis becoming a notifiable disease

After the Ministry of Health, Sri Lanka, declared leishmaniasis as a notifiable disease in 2008, the Epidemiology Unit was given the task of case detection through their well-established vertical surveillance system, and the AMC was given the task of sandfly vector control. In 2019, a national circular on "Guidelines on Prevention and Control of Leishmaniasis"[79] was issued by the Ministry of Health, Sri Lanka . However, in spite of having two management bodies as well as national circulars and conducting case surveillance to a certain degree, the CL case numbers continued to increase,[8] probably due to inadequate coordination between the two management bodies. Also, a recent study had reported inadequate notification; in this study, a survey conducted among 188 Medical Officers cited unavailability of notification forms, heavy workload, and inadequate supportive staff as the reported barriers for notification by the Medical Officers.[80] Due to rising CL case numbers, the Ministry of Health, Sri Lanka, identified the AMC as the focal point for control of leishmaniasis in August 2022, authorizing the AMC to handle both case surveillance and vector surveillance and control aspects, bringing control of leishmaniasis under one roof. Furthermore, since Sri Lanka reports more than 3000 new CL cases/year over the last 6 years consecutively (2018–2023) and the cumulative CL incidence is > 30, 000 from 2009 to 2024,[8] a WHO-funded first 5-year National Strategic Plan (NSP) for prevention and control of leishmaniasis 2024–2028 was developed by a team of experts selected by the WHO through an open bidding method requesting for proposals.[81]

Development of the national strategic plan for prevention and control of leishmaniasis in Sri Lanka 2024–2028

During the development of the NSP, the team of consultants selected by the WHO performed a situational analysis, and strengths and gaps were identified. For this process, the team performed an in-depth literature review, visited four of the five highest CL incidence-reporting districts and dermatology units in those hospitals, and met the stakeholders (dermatologists, academics from universities currently diagnosing leishmaniasis, clinicians, histopathologists, microbiologists, epidemiologists, hospital administrators, laboratory staff, CL patients, and villagers). In addition, the epidemiology unit, AMC (the focal point), and the Medical Supplies Division, which is responsible for the management of drug supply chain of the Ministry of Health, were also consulted. The strengths identified included having a vertical surveillance system in Sri Lanka, having a "Leishmaniasis case management guideline" developed in 2013 by the Sri Lanka College of Dermatologist;[54] having MLTs and PHLTs in the Ministry of Health, who are used to collect samples from leprosy and malaria patients (when malaria was prevalent in the country); having an entomology unit and entomologists and entomology assistants (EAs) at the AMC Headquarters and in the periphery; having a central drug management system under the Ministry of Health, Sri Lanka; and having performed important research activities in leishmaniasis over the years. However, the team identified many gaps during the review process; the notification system for leishmaniasis is not strong enough, having only a passive case detection system and absence of an active house-to-house case detection system even in the high CL prevalence areas; leishmaniasis case management guidelines needed updating and needed to be re-named as "National Guidelines"; MLTs and PHLTs were found to be *not* properly trained at national level for sample collection from CL lesions and to carrying out Giemsa staining of SSS; entomologist and EAs are not trained properly for sandfly collection, sandfly identification, and investigate sandfly bionomics and insecticide susceptibility, although few small-scale studies are available in the literature. Absence of a leishmaniasis reference laboratory and availability of PCR and culture being limited to only a few university laboratories were some of the key gaps identified. Also, absence of a national surveillance plan to screen for animal reservoir and frequent stockout of SSG and unavailability of any other WHO-prequalified drugs to treat CL and VL had also been identified as key gaps.[57]

Based on the situation analysis, the expert committee had identified the overarching goal as "To control cutaneous leishmaniasis for possible elimination as a public health problem in the future and prevention of VL and MCL" with two objectives to achieve the goal: to reduce the annual incidence of CL < 5 per 10,000 population by 2028 (approximately 6600 cases) and to ensure zero mortality due to VL.[57] To achieve the objectives, the NSP describes three strategic interventions: leishmaniasis surveillance including for CL, VL, and MCL, case diagnosis and management, and integrated vector management. The NSP further describes five supporting areas for the three strategic plans: leadership, program governance and management, community awareness and engagement on prevention and care, quality assurance, capacity building, and operational research.[57] The NSP has identified the monitoring and evaluation methods and indicators and describes the activity plan for 5 years with quarterly breakdowns and timelines. A mid-term review had been suggested by the 2nd quarter of 2026. The NSP also identified key challenges: CL being an NTD, financial commitment by the government, leadership, sustainability, and implementation of an integrated approach.[57]

DISCUSSION

Sri Lanka is an endemic country for CL now reporting CL over 3 decades, having a cumulative CL case of over 30,000 since 2009 and reporting over 3000 cases/year within the last 6 years.[8] We have also reported few cases of visceral and mucosal forms in an *ad hoc* manner. Although leishmaniasis was listed as one of the notifiable diseases in the country since 2008 and having certain strengths in the healthcare system, there were gaps in the notification and case surveillance systems.[80] Similarly, there were gaps in the diagnostic aspects due to lack of proper training of the laboratory staff, lack of a reference laboratory, and having only a few PCR laboratories. There was no proper training for entomologists on sandfly identification, and no guidelines were available for sandfly control. Sandfly bionomics and insecticide susceptibility were also not known adequately. More work is needed in vector incrimination and sandfly surveillance as well. Lack of information on reservoir host and frequent stockout of anti-leishmanials were also reported. Therefore, a recently carried out joint malaria, filariasis, leishmaniasis, and dengue program review 2024 conducted by the WHO had recommended to strengthen and sustain competencies in the surveillance system, enhance skills and capacities of healthcare workers, digitalize parasitological, entomological, and clinical data, strengthen governance and program management, enhance advocacy, risk communication, and community engagement, and strengthen cross-border collaboration.[82]

The program to eliminate VL as a public health problem from in Asia with regional strategies began in 2005. The countries included India, Bangladesh, and Nepal, where 70% of the global VL cases were reported and a memorandum of understanding was signed between the three countries.[83] On the 31st of October 2023, the WHO announced that Bangladesh had successfully eliminated VL as a public health problem (annual case incidence less than 1/10,000 population) and became the first country to do so.[84]

Currently, the prevalent species in Sri Lanka causing CL is *L. donovani,* and the abundant potential vector is *P. argentipes*.[85] Both parasite and vector species in Sri Lanka are genetically closely related to the species in the region than to other geographic regions in the world.[34,37,63] Therefore, it is important to implement a CL elimination program in Sri Lanka when considering elimination of VL from the region as genetic mutations in the SL-CL *L. donovani* or in Sri Lankan *P. argentipes* can provoke VL upsurges in the region and may lead to reemergence of VL in the region. Identifying this need, as well as due to the persistent rise of CL case numbers (> 3000/annum) over the last 6 years, the WHO-mediated first NSP for prevention and control of leishmaniasis in Sri Lanka 2024–2028 has now been drawn, and implementation had been initiated by the focal point AMC. Since Sri Lanka has an excellent track record of elimination of lymphatic filariasis and malaria, we are optimistic that together with community engagement, the Ministry of Health could successfully achieve the goal of elimination of leishmaniasis as a public health problem from Sri Lanka by 2028.

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Conflicts of interest
There are no conflicts of interest.

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Original Article

Protein-energy malnutrition and micro-nutrient deficiencies: Possible culprits in susceptibility and severity of visceral leishmaniasis

B. M. Younis[1], A. M. Musa[1], A. M. Abdelraouf[1], W. S. E. Saeed[1], M. A. Saeed[1], M. A. Magzoub[2], A. A. Beshir[3], M. E. E. Elfaki[4], Elyazeed N. Suliman[5], M. A. Awad Eljeed[1], A. J. Suleiman[1], E. A. G. Khalil[1]

[1]Institute of Endemic Diseases, University of Khartoum, [2]Garden City University, [3]Faculty of Medicine, University of Khartoum, Khartoum, Sudan, [4]Faculty of Medicine, King Khalid University, Abha, Saudi Arabia, [5]Faculty of Medicine, Al-Neelain University, Khartoum, Sudan

Abstract

Visceral leishmaniasis (VL) is a widespread highly morbid tropical infection. Protein-calorie and micro-nutrient deficiencies contribute to susceptibility and disease severity. This study aimed to delineate the effects of protein-calorie malnutrition, selenium (Se), zinc (Zn), and copper (Cu)] level derangements on immune responses in VL patients. Following informed consent, 77 sequential parasitologically confirmed VL patients and 112 apparently healthy controls were recruited. Weight for height Z-score (WHZ), albumin, Se, Zn, and Cu were markers for protein-calorie and micro-nutrient disturbances. Leishmanin skin test (LST), direct agglutination test (DAT), and IL-10/TNF-α/IFN-γ levels in supernatants of soluble *Leishmania*–antigen whole blood-stimulated samples were measured as indicators of immune responses. VL patients have significantly lower baseline WHZ levels compared to controls (−2.2 ± 1.1 and −1.4 ± 1.3, respectively, $P = 0.0006$). Albumin levels were similarly reduced (2.9 and 3.4 g/dL, $P = 0.06$). Patients had marginally significant lower Se levels compared to controls (57.6 ± 13.1 and 61.7 ± 13.4 μg/L, respectively, $P = 0.04$). Pretreatment Se levels were significantly lower compared to post-treatment ones (56.9 ± 13.1 and 65.2 ± 22.1 μg/dL, respectively, $P = 0.02$). Zn levels were significantly lower in patients compared to controls (36.2 ± 17.3 and 72.9 ± 12.5 μg/L, respectively, $P = 0.0002$). Cu levels were four-fold higher in patients compared to controls [median 336 and 73.5 μg/L, respectively $P = 0.00001$]. The Cu:Zn ratio was significantly higher in patients compared to controls (9.6 and 1.0, respectively, $P = 0.0001$). LST was non-reactive in all VL patients with DAT levels >6400. Pre- and post-VL treatment levels of IFN-γ and IL-10 levels were comparable in patients and controls. Pretreatment TNF-α levels were significantly higher compared to post-treatment ones (median: 64.1 and 36.7 pg/mL, respectively, $P = 0.0002$). Five per cent (6/112, 5.4%) of the healthy controls developed VL during follow-up, and 15.2% (17/112) developed subclinical infection with LST conversion in 14/17 (82.4%, mean induration of 8.5 ± 2.6 mm). Three (3/17, 17.6%) converted in DAT and LST. Low WHZ, Se, and Zn levels with high Cu/Cu: Zn ratios probably increase susceptibility to VL.

Keywords: Micro-nutrient deficiencies, protein-calorie, susceptibility, visceral leishmaniasis

Address for correspondence: Prof. E. A. G. Khalil, Department of Clinical of Pathology and Immunology, Institute of Endemic Diseases, University of Khartoum, Khartoum 45235, Sudan.
e-mail: eltahirk@iend.org
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INTRODUCTION

Visceral leishmaniasis (VL) is a systemic disease caused by *Leishmania donovani* and *Leishmania infantum,* in the Old World. The disease commonly presents with symptoms of fever, malaise, cough, abdominal pain, diarrhea, epistaxis, hepato-splenomegaly, pancytopenia, lymphadenopathy, and weight loss. The incubation period is between 2 and 6 months, but it may be longer. Non-immune individuals traveling in VL-endemic areas become infected with a more acute course. A considerable number of *Leishmania-infected* individuals in the endemic areas develop subclinical infections. At-risk populations include children, immune-compromised, and undernourished individuals. The vector, *P. orientalis* inhabits areas where *Acacia seyal* and *Balanites aegyptiaca* vegetation are abundant. *Leishmania* isolates from Sudan belong to the *L. donovani* species and probably show anthroponotic transmission. Immunity to leishmaniasis is mediated by both innate and adaptive (T cells) responses.[1-9] VL could be fatal if not treated, with more than half of successfully treated patients developing Post Kala-azar Dermal Leishmaniasis (PKDL), a dermatosis, which represents part of the spectrum of VL and a probable reservoir.[3,10-15]

Macro-nutrients (carbohydrates, proteins, and fats), as well as micro-nutrients (water-soluble vitamins, fat soluble vitamins, calcium, potassium, sodium, iron, zinc, copper, and selenium), are important requirements for growth, tissue repair, and defense against infection. Malnutrition is widespread throughout the developing world, with acute and chronic forms contributing directly or indirectly to disease morbidity and mortality through weakened body defense mechanisms.[16-19] It is estimated that more than two billion people in the world have deficiency in one or more key micro-nutrients (vitamin A, iodine, iron, zinc, and folate).[20] Micro-nutrient deficiencies have become increasingly important since it led to several health problems such as impairment in growth, lower cognitive responses, and immune system defects. In addition, they are responsible for more than a third of deaths in children under 5 years.[21] The high prevalence of bacterial and parasitic diseases in developing countries results in increased malnutrition, and similarly malnutrition increases susceptibility to and severity of infections.[18, 22]

MATERIALS AND METHODS

This was a longitudinal, community-based and case–control study that was conducted in VL-endemic areas in Gedarif State, Eastern Sudan. Following ethical and scientific approvals, informed consent was obtained from the parents/guardians of participants. Sample size was estimated as 77 cases and 112 healthy controls (power of 80%, confidence interval (CI) of 95%). Seventy-seven sequential and parasitologically confirmed VL patients and 112 healthy controls of the age groups 3 to < 18 years were recruited. Individuals with chronic diseases and those reactive for HBV, HCV, HIV, leishmanin skin test (LST), and direct agglutination test (DAT) were excluded. Clinical and demographic data were collected at recruitment/diagnosis in specially designed forms. Standard scales and calipers were used to measure weight and height. Weight for height ratio was calculated using the CDC growth calculator software.[23] Weight for height Z-score (WHZ) and albumin levels were measured as indicators of protein-calorie malnutrition. Sera levels of Se, Zn, Cu, and Cu: Zn ratios were measured as indicators of micronutrient status. LST, DAT and levels of IL-10/TNF-α/IFN-γ in the supernatants of soluble leishmanial antigen-stimulated whole blood were measured as indicators of humoral and cell-mediated immune responses. VL patients were treated with paromomycin plus sodium stibogluconate for 17 days. VL patients were followed-up to the end of treatment (17 days), while the healthy controls were followed-up for 1 year for reviewing the development of VL.

Leishmanin reagent (Pasteur Institute, Tehran, Iran) was injected intra-dermally into the volar aspect of the right arm 4 centimeter (Cm) below the ante-cubital crease. Tests were read after 48–72 h using the ballpoint pen technique, and indurations of ≥5 mm were taken as reactive.[3, 24] DAT was performed as previously reported.[25] Soluble *Leishmania* antigens (sLA) were prepared from promastigote cultures of *L. donovani* in the stationary phase as previously.[26] The supernatants were divided into aliquots and stored at −80°C till use. The protein content was quantified using the Pierce BCA Protein Assay Kit (Bio-Rad, USA). Whole blood stimulation was performed as follows: blood samples were collected in heparinized tubes. Aliquots of 1 mL of whole blood were incubated in tubes with 10 μg/mL sLA, and a second set of tubes were stimulated with phytohemagglutinin (PHA) 40 μg as positive controls. A third set of tubes with no additives were set as negative controls. Tubes were incubated at 37°C for 24 h, followed by centrifugation at 2000 *g* for 10 min. The supernatants were collected and stored at -20°C for cytokine analysis.[26] The concentrations of IL-10, IFN-γ, and TNF-α in supernatants were measured by ELISA assay using *Koma Biotech ELISA* Kits, Seoul, Korea). Plasma Zn and Cu were measured colorimetrically using an automated biochemistry analyzer (BioSystems Barcelona, Spain). Flame atomic absorption spectrometer was used for the determination of plasma

Se levels. Samples were prepared and run following the manufacturer's instructions (Buck Scientific model 210 VGP, USA). Photometer 5010 model V 5+ was used for measurement of the albumin level following the manufacturer's instructions (Photometer 5010 model V 5+ Germany).

Data collection and analysis

Data were extracted and double entered in electronic case record forms. Data cleaning was done, and logical numbers (no data, negative number, and blank item) were rechecked. The original case record forms were explored again when necessary. Data were analyzed using the EpiInfo7 software.

RESULTS

Demographic data of the study population

One hundred eighty-nine individuals of the age group 3 to <18 years participated in the study with a mean ±SD age of 7.6 ± 3.9 years and a male: female ratio 1.3. VL patients [77/189, 40.7%] and the healthy controls [112/189, 59.3%] were comparable for mean ages (8.1 ± 3.5 and 7.1 ± 3.5 years, respectively, $P = 0.06$) [Table 1].

Baseline nutritional status of the study population

Baseline WHZ scores were significantly lower in VL patients compared to apparently healthy controls (mean ±SD of −2.2 ± 1.1 and −1.4 ± 1.3, respectively, $P = 0.0006$). Similarly, mean Zn levels were significantly lower in VL patients compared to controls (36.2 ± 17.3 and 72.9 ± 12.5 µg/L, respectively $P = 0.0002$). However, Cu levels in VL patients were fourfold higher than that of the controls (median 336 and 73.5 µg/L, respectively, $P = 0.00001$). In addition, the Cu:Zn ratio was significantly higher in VL patients compared to controls (9.6 and 1.0, respectively, $P = 0.00001$). There were no significant differences between VL patients and controls for age and Se level (8.1 ± 3.5 and 7.1 ± 3.5 years, $P = 0.06$; 57.6 ± 13.1 µg/L and 61.7 ± 13.4 µg/L, $P = 0.1$) [Table 1]

The nutritional status of the VL patients before and after treatment

WHZ, Zn, Se, and albumin levels in VL patients were significantly lower before treatment compared to levels after treatment, with mean ±SD values of −2.2 ± 1.1 and −1.6 ± 1 ($P = 0.005$); 36.2 ± 27.3 and 51.0 ± 28.4 µg/L ($P = 0.0001$); 56.9 ± 13.1, 65.2 ± 22.1 µg/L ($P = 0.02$) and 29.2 ± 6.5 and 33.9 ± 6.3 g/L ($P = 0.0006$). However, patients' Cu levels before and after treatment were fourfold greater than those of controls (mean, 75.1 ± 12.7; median of 336 µg/L). Although treatment significantly reduced Cu levels (pretreatment level 336 µg/L; post-treatment level 249.1 µg/L, $P = 0.004$), no significant change was observed in the Cu/Zn ratio (pre-treatment 9.6 and post-treatment 6.7, $P = 0.4$) [Table 2].

VL patients' IFN-γ and IL-10 levels were comparable in pre and post-treatment samples (IFN-γ: median 14.4 and 14.7 pg/mL, respectively, $P = 0.4$; IL-10: median 11.1 and 5.1 pg/mL, respectively, $P = 0.1$). On the other hand, TNF-α levels were significantly higher in pretreatment samples compared to post-treatment ones (median 64.1 and 36.7 pg/mL, respectively, $P = 0.0002$). More than half of VL patients (43/77, 55.8%) had positive DAT at diagnosis,

Table 1. Baseline characteristics of the study participants by age and nutritional status

Characteristic	Age	WHZ	Zn µg/L	Median Cu µg/L*	Cu:Zn ratio	Se µg/L
Total study population (*n* = 189)	7.6 ± 3.9	−1.6 ± 1.3	62.8 ± 24.1	85	2.6	60.6 ± 13.4
Visceral leishmaniasis (VL) patients (*n* = 77)	8.1 ± 3.5	**−2.2 ± 1.1**	**36.2 ± 17.3**	**336**	**9.6**	57.6 ± 13.1
Healthy controls (*n* = 112)	7.1 ± 3.5	**−1.4 ± 1.3**	**72.9 ± 12.5**	**73.3**	**1.0**	61.7 ± 13.4
P value	*0.06*	*0.0006*	*= 0.0002*	*=0.00001*	***0.00001***	*0.1*

Continuous data are expressed as mean ±SD;
*median was used for statistical calculation. The mean difference is significant at $P < 0.05$

Table 2. Immunological and nutritional characteristics of the VL patients at pre-treatment and post-treatment

Characteristic Mean ± SD	IFN-γ (pg/mL)*	IL-10 (pg/mL)*	TNF-α (pg/mL)*	WHZ	Zn (µg/L)	Cu (µg/L)*	Cu/Zn ratio	Se (µg/L)	Albumin (g/dL)
Pretreatment *n* = 77	14.4	11.1	64.1	−2.2 ± 1.1	36.2 ± 27.3	336	9.6	56.9 ± 13.1	29.2 ± 6.5
Post-treatment *n* = 77	14.7	5.1	36.7	−1.6 ± 1.1	51.0 ± 28.4	249.1	6.7	65.2 ± 22.1	33.9 ± 6.3
P value	***0.4***	***0.1***	***0.0002***	***0.005***	***0.0001***	***0.004***	***0.4***	***0.02***	***0.0006***

Continuous data are expressed as mean ±SD
*= the median used for calculation, statistical significance at $P < 0.05$

Table 3: Characteristics of individuals who progressed to VL and the leishmanin skin test (LST)-convertors among the control group

Characteristic	Age	WHZ	Zn μg/L	Cu μg/L	Cu/Zn ratio	Se μg/L
LST converters (*n* = 17)	6.5 ± 3.3	−1.2 ± 1.3	77.5 ± 8.9	72.6 ± 11.7	0.9	57.8 ± 12.8
Progressed to VL (*n* = 6)	6.7 ± 3.6	−1.8 ± 0.9	66.8 ± 2.6	73.5 ± 8.6	1.1	49.2 ± 5.1
P value	***0.8***	***0.2***	***0.03***	***0.4***	***0.9***	***0.002***

Continuous data are expressed as mean ±SD. The mean difference is significant at $P < 0.05$

and more patients (47/77, 61.0%) became positive in the post-treatment period [Table 2].

Baseline characteristics of the healthy controls who progressed to VL

Five per cent (6/112, 5.4%) of apparently healthy controls developed VL, while the rest (106/112, 94.6%) remained disease-free to the end of the 1-year follow-up. Zn and Se levels were significantly lower in those who progressed to VL compared to the rest (Zn mean ±SD of 66.8 ± 2.6 and 73.8 ± 11.2 μg/L, respectively, $P = 0.0002$) and mean ±SD Se of 49.2 ± 5.07 and 62.3 ± 13.4 μg/L, respectively, $P = 0.004$). However, the two groups were comparable with respect to age (6.7 ± 3.6 and 7.3 ± 3.6 years, respectively, $P = 0.4$), WHZ (−1.8 ± 0.9, −1.4 ± 1.4, respectively, $P = 0.7$), and Cu levels (73.5 ± 8.6 and 75.2 ± 12.9 μg/L, respectively, $P = 0.6$). Cu/Zn ratios were not different in the two groups (ratios of 1.1 and 1.0, respectively).

Baseline characteristics of the leishmanin-converters and non-converters among apparently healthy controls

Fifteen per cent of the healthy control (17/112, 15.2%)) converted to positive LST, and three of them were positive for both LST and DAT. The Cu/Zn ratio was comparable between the leishmanin-converted group and the leishmanin non-reactive group (95/112, 84.8%), with a ratio of 0.9 and 1.0, respectively. In addition, the two groups were comparable for age, WHZ, Zn, Cu, and Se with a mean ±SD of 6.5 ± 3.3 years, −1.2 ± 1.3, −0.9 ± 1.6, 77.5 ± 8.9 μg/L, 72.6 ± 11.7 μg/L, and 57.8 ± 12.8 μg/L in the leishmanin-converted group and 7.1 ± 3.6 years, −1.5 ± 1.3, −0.9 ± 1.7, 72.7 ± 11.2 μg/L, 75.6 ± 12.9 μg/L, and 62.3 ± 13.5 μg/L in the non-converted group, respectively ($P = 0.3, 0.4, 0.8, 0.06, 0.3$, and 0.2, respectively). The LST-converted group had significantly higher Zn and Se compared to the group that progressed to VL (6/112; 5.3%) with mean Zn: ±SD of 77.5 ± 8.9 μg/L and Se 57.8 ± 12.8 μg/L in the LST-converted group and Zn: 66.8 ± 2.6 μg/L and Se: 49.2 ± 5.1 μg/L in the group that progressed to VL ($P = 0.03$ and 0.002, respectively). However, no difference was observed concerning the Cu/Zn ratio in the leishmanin-converted group compared to the group that progressed to VL, with ratios of 0.9 and 1.1, respectively. Furthermore, no difference was observed between the two groups concerning age, WHZ, and Cu levels, with the mean ±SD of 6.5 ± 3.3 years, −1.2 ± 1.3, and 72.6 ± 11.7 μg/L in the LST-converted group and 6.7 ± 3.6 years, −1.8 ± 0.9, and 73.5 ± 8.6 μg/L in the group that progressed to VL ($P = 0.8, 0.2, 0.4$, and 0.4, respectively) [Table 3].

The nutritional status of apparently healthy controls who progressed to VL compared to VL patients

Zn was significantly lower in VL patients[27] compared to the apparently healthy controls who progressed to VL (6/112), with mean ±SD of 36.2 ± 27.3 and 66.8 ± 2.6 μg/L, respectively ($P = 0.00001$). However, Se levels were significantly higher in VL patients compared to the apparently healthy controls who progressed to VL, with mean ±SD of 56.8 ± 13.1 and 49.2 ± 5.1 μg/L, respectively ($P = 0.01$). Cu was fourfold higher in VL patients compared to apparently healthy controls who progressed to VL, with a median of 336 μg/L in VL patients and 71.5 μg/L in the controls who progressed to VL ($P = 0.0001$). No significant difference was observed between the two groups in the Cu/Zn ratio (9.6 and 1.1, respectively, $P = 0.5$). Meanwhile, age and WHZ were not different between the two groups, with mean ±SD of 8.1 ± 3.5 years and -2.2 ± 1.1 in VL patients and 6.7 + 3.6 years and −1.5 ± 1.6 in the controls who progressed to VL ($P = 0.1, 0.3$, and 0.6).

DISCUSSION

The nutritional status of individuals infected with *Leishmania* spp. plays an important role in the clinical course of the disease and is a major determinant of progression and severity. Current data indicated that patients with active VL have significantly reduced weight, albumin, Zn, and Se with significantly high Cu and Cu/Zn ratios. These findings are in agreement with previous reports, while the persistence of high Cu levels is discordant with that observed in previous studies.[27-29] High serum Cu levels promote the Th2 type of immune responses with production of IL-10 and suppression of Th1 responses. In addition, low serum albumin further favors low Zn

and could further downregulate the immune responses and contribute to increased susceptibility and severity of infections, as was reported previously.[18, 22, 30-32] Nutritional deficiencies among individuals in the VL-endemic area in Sudan could explain the reported low subclinical: clinical ratio compared to neighboring African countries. This finding was discordant with our finding of higher subclinical: clinical ratio.[2, 5, 33,34] Increased body weight following successful VL treatment is not concordant with previous reports.[28] Our study proved that a significant association exists between decreased weight for height Z-score in VL patients, which is in agreement with previous reports.[27, 35] Significantly low serum Zn in VL patients is expected since patients' diet in the studied endemic area is low in animal products and high in phytates, as previously reported.[29, 36,37]

Less is known about the physiological function of Cu, with few reports indicating an antioxidant activity. Significantly high serum Cu levels and high Cu/ZN ratio are concordant with those of previous studies that documented high plasma Cu levels in VL patients, reaching levels which have been shown to be toxic *in vitro*. Previous studies demonstrated a highly significant positive correlation between plasma Cu and parasite-specific IgG in patients, suggesting that high plasma Cu might interfere with anti-leishmania immune responses, leading to non-protective Th2 humoral immune responses. High plasma Cu level may result in deposition of Cu in body tissues including lymph nodes, leading to toxicity and disruption of lymph node barriers, further increasing susceptibility and progression.[37-39] Low Zn was found to be strongly associated with progression to VL among our apparently healthy controls, which is in agreement with previous reports.[40] Se deficiency and susceptibility to disease were not well-studied, but it is well documented that Se deficiency leads to reduced glutathione peroxidase (GSH-Px) enzyme activity with decreased ability to degrade H_2O_2. To our knowledge, our study is the first to examine Se levels in Sudanese VL patients. We have shown that both healthy endemic controls and VL patients have low Se levels, which could be due to poor diets that are prevalent in VL-endemic areas in Sudan. In the present study, we have shown that low Se and Zn status at the time of infection is associated with progression to clinical disease, which is further supported by reports of previous studies.[37, 41-43] Persistently low Zn and Se levels in VL patients could possibly affect treatment outcomes, in agreement with previous reports.[44] IFN-γ and IL-10 levels post-treatment did not change significantly, probably indicating the slow process of immune reconstitution, which probably may take up to 2 years to complete.[3, 45] On the other hand, TNF-α levels increased significantly during treatment, with significant reduction after treatment, which is in agreement with previous reports, presenting it as an important prognostic marker.[46,47] The DAT reports did not change with treatment, but indicated an upward trend toward the end of treatment. Previous data from Sudan indicated that leishmania antibodies may remain positive in some patients for more than a year.[3] Based on these results, a high protein diet with Zn and Se supplements should be part of the dietary supplements for VL patients in hospitals and healthy children in the endemic regions to improve treatment outcomes and reduce VL incidence.

CONCLUSION

Low WHZ score, low Zn, and low Se with a high Cu and Cu/Zn ratio are probably important risk factors for VL. TNF-α levels can be used as a prognostic factor for treatment monitoring. Higher Zn and Se levels probably favor subclinical status over development of overt disease.

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Conflicts of interests
There are no conflicts of interest.

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Original Article

Liposomal amphotericin B (AmBisome®) pharmacokinetics: Reaching the skin, review of the literature, and a case series of treated severe disfiguring post kala-azar dermal leishmaniasis

Eltahir Awad Gasim Khalil, B. M. Younis, W. S. E. Seed, M. A. Magzoub[1], A. M. Elhassan, A. M. Musa

Institute of Endemic Diseases, University of Khartoum, [1]Garden City University, Khartoum, Sudan

Abstract

Post kala-azar dermal leishmaniasis (PKDL) that usually follows successfully treated visceral leishmaniasis (VL) can be severe and disfiguring. Treatment with sodium stibogluconate is prolonged, expensive with colossal cardiorenal toxicities. Early trials with liposomal amphotericin B (AMB) (AmBisome®) revolutionized PKDL treatment with increased safety and improved efficacy. AmBisome® pharmacokinetics and dynamics are poorly understood. This study aimed to show the efficacy of AmBisome® through its permeation within skin lesions. Following guardians' written consent, ten PKDL patients with a history of parasitolgically confirmed VL, typical histopathological patterns, and presence of *Leishmania* antigens/parasites were enrolled. Blood samples were taken for DAT, hematological, and biochemical investigations. Leishmanin skin test (LST) was performed, and skin biopsies were taken for histopathology and electron microscopy. Patients received 14 daily injections of AmBisome® at 3 g/Kg/body weight. Skin biopsies revealed hyperkeratosis, loose epithelioid granulomas with dermis lymphocytic and histiocytic infiltrates, destruction of the basal layer, melanin incontinence, and loss of basal layer pigmentation. *Leishmania* antigens and scanty parasites were detected in the lesions. LST was non-reactive in all patients. DAT titers ranged from 6400 to 51,200. Patients responded markedly; healing continued for a few months after the stoppage of the drug. Electron micrographs showed skin lesion infiltration by T lymphocytes, macrophages loaded with parasites, and lipid droplets. Liposomal AMB was likely absorbed by circulating macrophages though their high-density lipoprotein receptors to be recruited to the skin, where the leishmanial parasites will be engulfed as well. Fusion of the phagosomes of the drug and that of the parasite leads to local release of the drug, which combined with the ergosterol of the parasite cell membrane led to its demise. Skin lesion healing continued after stoppage of the drug due to the drug persistence in macrophages. In conclusion, liposomal AMB enters the skin through recruited circulating macrophages with parasite phagocytosis and killing through binding with cell membrane ergosterol and pore formation.

Keywords: AmBisome®, *Leishmania* parasite, parasite wall ergosterol, post kala-azar dermal leishmaniasis

Address for correspondence: Prof. Eltahir Awad Gasim Khalil, Department of Clinical Pathology and Immunology, Institute of Endemic Diseases, University of Khartoum, P. O. Box 45235, Khartoum, Sudan.
E-mail: eltahirk@iend.org

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BACKGROUND

For more than 70 years, broad-spectrum anti-fungal polyene macrolides have been pivotal for invasive fungal infections with excellent clinical effects, despite their considerable cardiorenal toxicities. Great efforts went into the reformulation of these drugs with the development of lipid formulations, leading to improved tolerance and marked reduction in side effects. The new liposomal formulations lead to re-purposing of the drugs so that it can be an important *aide* in the treatment of important neglected parasitological diseases such as visceral leishmaniasis (VL). Three lipid amphotericin B (AMB) formulations have been developed over time: AMB lipid complex (ABLC), liposomal AMB, and colloidal dispersion AMB, which was later discontinued due to its severe side effects. The reduced toxicity is mainly due to lack of high-density lipoprotein receptors in renal tissues, with the drugs mainly targeting the RE cells, especially the macrophages, the principal cell type in VL immunology. Despite development of second-generation anti-fungals (triazoles and echinocandins), AMB lipid formulations remain the most widely used due to their wide spectral effects, lowest resistance rates, and its intra-thecal use for fungal meningitis.[1-8] Although liposomal AMB formulations are relatively safe, they still induce some infusion-related side effects such as headache, fever, chills, hypotension, malaise, muscle and joint pains, stiffness, slight increase in liver enzyme levels, renal and heart-lung toxicities, and gastro-intestinal symptoms. These side effects are thought to be brought by cytokine release and can partially be prevented by premedication with antihistamines, analgesics, and corticosteroids.[9-11] On the other hand, idiosyncratic reactions manifesting as chest/abdominal pains and dyspnea could probably be caused by the liposomes in the formulation.[12,13] The drug side effects happen when AMB binds to the cholesterol of the mammalian cell plasma membrane, leading to damage of the kidney, heart, and blood cells.[14-17] Recent guidelines suggest that although LAMB, AMB-deoxycholate (D-AMB), and ABLC have the same spectral activity, they have different frequencies of side effects.[18]

Liposomal AMB (e.g., AmBisome®, Gilead Sciences, Foster City, CA, USA) acts by fungal cell wall destruction, briefly as follows: the added deoxycholate in phosphate buffer increases AMB solubility in water and increases the stability of the suspension with binding of AMB to the ergosterol of the cytoplasmic membrane of the fungal cell, forming pores, leading to extravasation of electrolytes, proteins, and glucose from the fungal cells, leading to its lysis.[19]

Although liposomal AMB is now widely used in clinical practice, the pharmacodynamics and pharmacokinetics are poorly understood. AMB is poorly absorbed by the gastrointestinal tract, and it has to be given parenterally.[20] In addition, the pharmacokinetics varies considerably between different AMB lipid formulations (D-AMB and LAMB). Animal and human studies showed that different AMB formulations yield different concentrations in different body compartments and that the inactivated drug is slowly excreted by the kidneys.[21] LAMB shows a higher concentration in different reticulo-endothelial (RE) cells and organs (liver and spleen) and the blood.[22-26] Most of LAMB remains in the spleen and liver, where it declines in the renal tissues and lungs.[27] These differences in concentrations in different tissues are probably due to differences in binding of the liposome component and its binding to the plasma proteins.[28] Under unfavorable conditions (temperature above 50°C–100°C, light and low pH), LAMB is easily degradable.[29-31]

Liposomal AMB has been tested for its efficacy in VL immune-competent individuals and PKDL in Sudan in the late 1990s,[32,33] since then, its use at different doses increased exponentially for HIV/VL co-infections as well.[34-37] The use of liposomal AMB for the treatment of post kala-azar dermal leishmaniasis is well-established with marked reduction in treatment duration and cost.[38-40]

This study aims to demonstrate the efficacy and presence of LAMB in the skin of patients with severe PKDL through the migrations of macrophages to the skin lesions, throwing light in the elusive pharmacokinetics of LAMB.

MATERIALS AND METHODS

Ethical considerations

The study proposal was scientifically and ethically approved by the Scientific and Ethics Committees of the Institute of Endemic Diseases, University of Khartoum. Guardians of patients were informed and provided written consent on behalf of their children for participation in the study.

Study population

Ten patients with PKDL based on parasitologically confirmed recent history of VL (before 6 months) with successful treatment, typical histopathological patterns, presence of *Leishmania* antigens/parasites in the skin sections, non-reactive Leishmanin skin test (LST), and positive DAT with titer >6400 were enrolled. Skin biopsies for two children were subjected to electron microscopy studies to determine pathological changes, cell recruitment, and presence of liposomal AMB in the skin. Demographic, clinical data were collected in a specially designed form

(CRF). Blood samples were collected for hematological/biochemical investigations in pretreatment and weekly to the end of treatment.

Skin biopsies, H and E histopathology, and leishmania antigen demonstration

Skin biopsies using 5-mm skin biopsy punches were obtained and divided into 10% formol saline and 4% glutaraldehyde solutions for H and E and electron microscopy (two biopsies), respectively. Detection of *Leishmania* antigens in skin lesions was done by an immuno-peroxidase stain method using an in-house immune serum prepared in *BALBc* mice. Briefly, the mice were injected with 100,000 *L. donovani* promastigotes in the foot pad, followed by intra-peritoneal injection of 0.5 million promastigotes 7 days later. The mice were sacrificed, and blood was collected after a week. As the negative control, *L. donovani* promastigote absorbed sera were used.

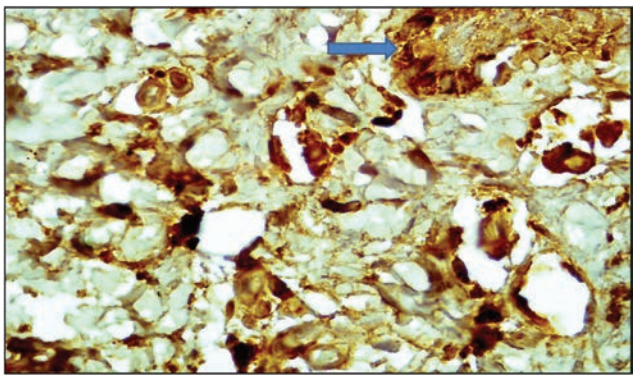

Figure 1: *Leishmania* antigens in the skin lesion basal layer (Immuno-peroxidase stain)

Leishmanin skin test and DAT

LST and DAT tests were performed as described previously (El-Hassan and Zijlstra 2001). An induration of ≥5 mm was considered reactive, while a DAT titer of >6400 was taken as positive.

RESULTS

The mean age of the children was 8.1 ± 4.3 with a male: female ratio of 2:1 with a skin lesion mean duration of 18.1 ± 10.6 months, while their PKDL grades were 3:3 in 6/10 and 1:3 in 4/10. Skin lesions were mainly nodulo-ulcerative and maculo-nodular. The leishmanin test induration was 00 mm (non-reactive in all patients 10/10). The DAT titers range from 6400 to 51,200. *Leishmania* antigens/parasites were reported in all biopsies [Figure 1] with hyperkeratosis, melanin incontinence and loss of melanin in the basal layer, dermal lymphocytic/macrophages, diffuse/solid epithelioid granulomas, and Langhans giant cells. Response to the treatment was gradual, and lesions started gradually by flattening and darkening of the nodular lesions, and the skin color gradually darkened. Lesions completely healed within 6 months with smoothing of the skin contour and restoration of normal color [Figures 2 and 3]. Electron micrographs showed presence of T-cells and macrophages with phagosomes with disintegrating parasites with lipid droplets [Figure 4].

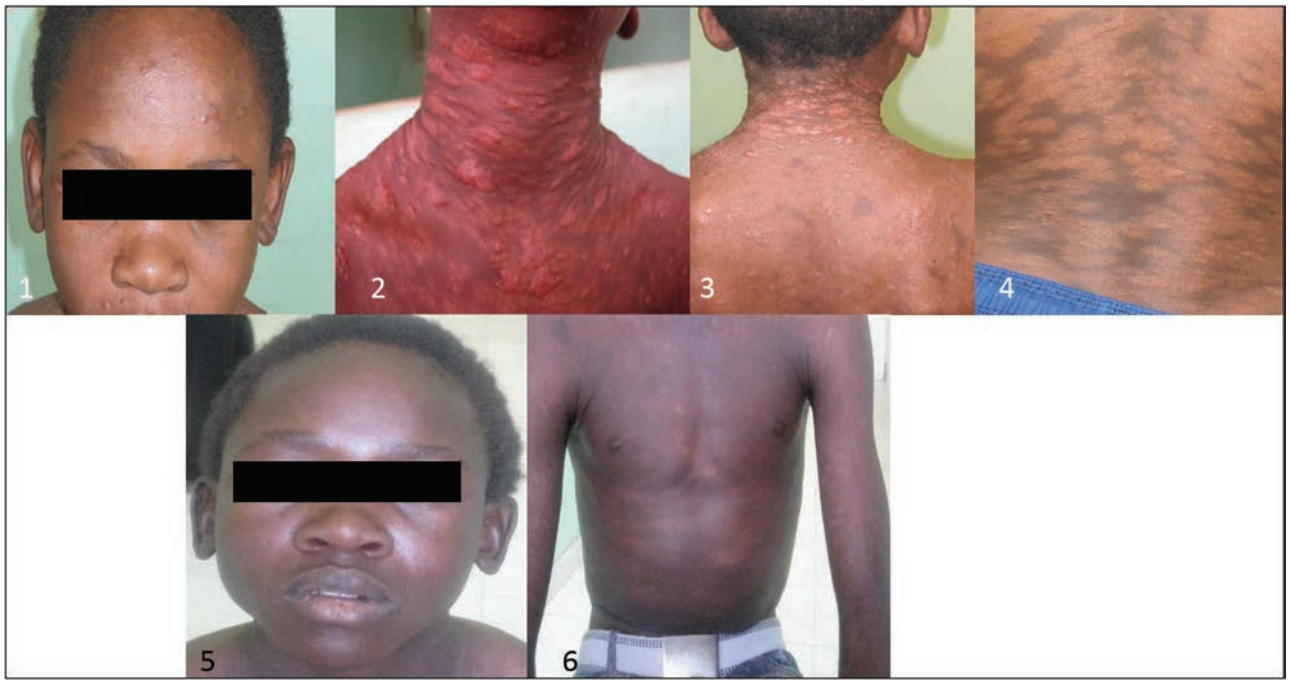

Figure 2: Patients RRR before and a year post treatment

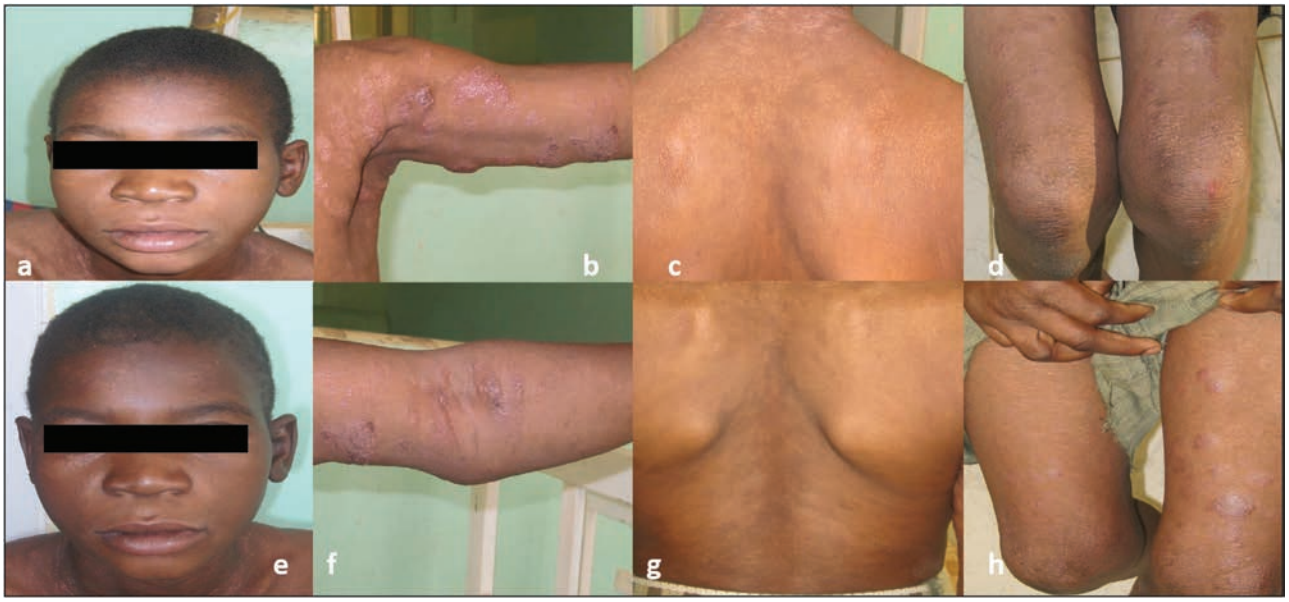

Figure 3: Patient DDD before and 6 months after treatment

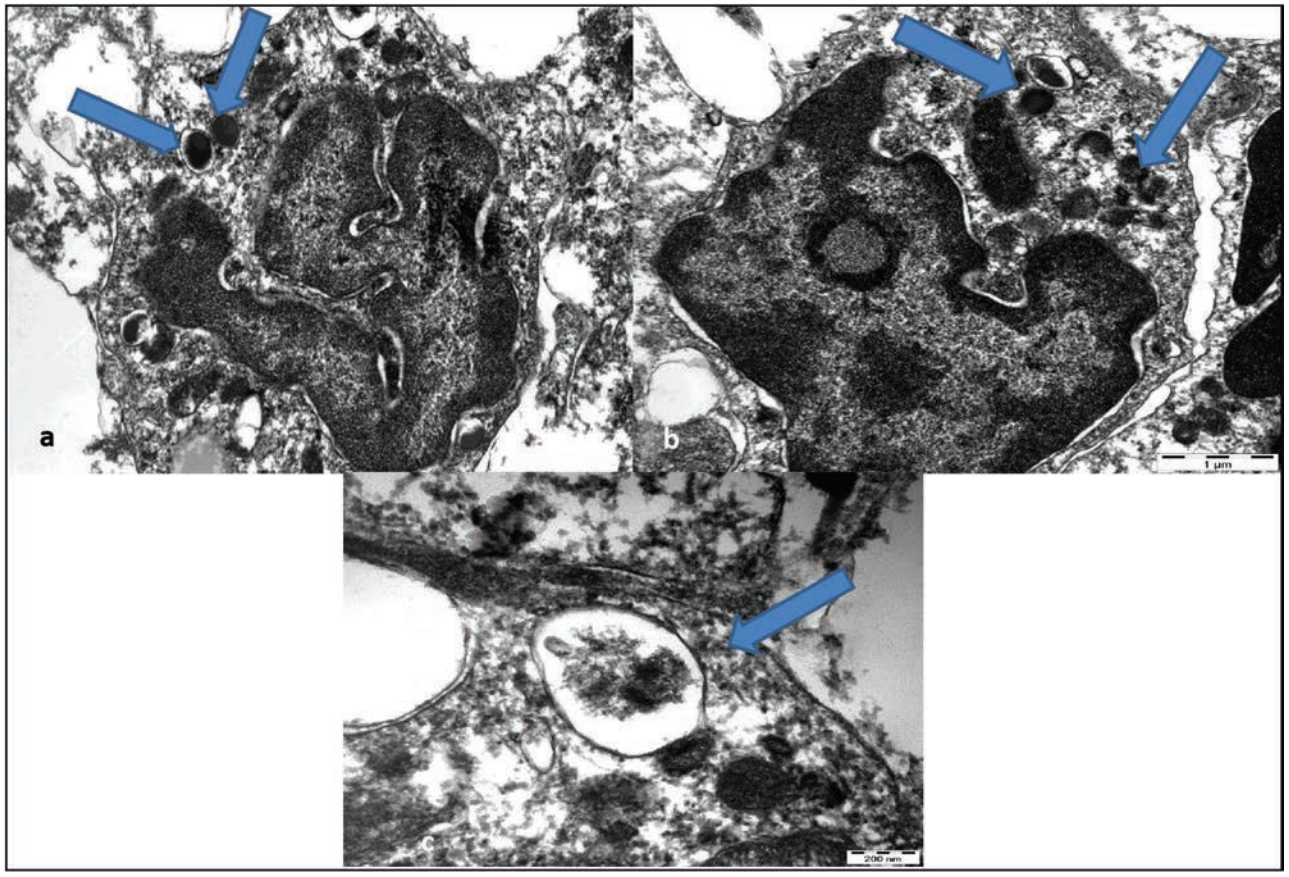

Figure 4: Electron microscopy micrographs: (a and b) Skin lesion showing a histiocyte with lipid droplets (c) a phagosome with disintegrating parasite

DISCUSSION

The use of liposomal AMB in the treatment of PKDL significantly reduced treatment duration and cost with reduction in the frequency of toxic effects. *Leishmania* parasite–macrophages could be detected in lymph nodes, bone marrows, and skin lesions of patients with PKDL. Parasites were more abundant in nodular skin lesions compared to macular ones.[41] Patients with chronic PKDL lesions have hypopigmented skin due to destruction of

the basal layer with loss of melanin pigmentation. PKDL skin lesions gradually healed and continued healing after stoppage of the drug, probably indicating the persistence of the drug in the skin after drug stoppages. PKDL skin lesions' healing following AmBisome® treatment is well-documented,[32,38,40] with minimum toxicities providing a circumstantial evidence that liposomal AMB reach to the skin. Reduced toxicities probably indicate that the drug/liposome complex rather than the free drug get to the skin lesions. Continuation of the healing process with restoration of skin tan after the rug stoppage probably indicates that the drug in the liposomal-bound form rather than the free drug is the cause. The presence of lipid droplets within the skin macrophages strongly indicates that liposomal AMB could get to the skin. This is probably due to blood macrophages/monocytes engulfing the liposomes/drug complex and then get recruited to the site of skin lesions, where they engulf *Leishmania* parasites, probably through the drug binding of the parasite's ergosterol, pore formation in the parasite membrane, and parasite lysis.[20]

In conclusion, liposomal AMB gets to PKDL skin lesions through macrophages with parasite killing in phagosomes through binding to parasite cell membrane ergosterol and parasite lysis.

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Conflict of interest

There are no conflicts of interest.

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