Research article

Morphometrics of amastigote forms of *Leishmania donovani* in cutaneous leishmaniasis patients in Sri Lanka: evidence for the presence of promastigote-like structures

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Abstract

Objective: Investigations have not yet been carried out to identify the different morphological forms of amastigotes present in patients with cutaneous leishmaniasis (CL) in Sri Lanka. Thus, this paper describes the existence of different amastigote forms in cutaneous lesions for the first time in Sri Lanka.

Methods: This was a retrospective study. One hundred and thirty skin smears were investigated to identify the different morphometric forms of *L. donovani*. In addition, demographic data (age, gender, occupation, household characteristics, and geographic area) were analyzed using department records.

Results: Of the 130 samples, 84 (60.83%) samples had amastigote forms. Three (2.31%) samples had amastigotes in intracellular locations while 43 (33.08%) had amastigotes extracellularly. Nineteen (14.62%) samples had amastigotes in intracellular and extracellular locations simultaneously. Promastigote-like structures (PLS) were found in 65 (50%) samples. Of the 65 samples, 19 (14.62%) had both PLS and amastigote forms. PLS alone (no association with amastigote forms) were found in 46 (35.38%) samples. Amastigotes were found predominantly in lesions <2 months old while PLS were more common in 8 to 12 months old lesions.

Conclusion: Microscopic examinations of skin smears revealed the presence of promastigote-like structures for the first time in patients with CL in Sri Lanka. Therefore, we suggest that different morphometric features of amastigotes should not be ignored as they may be useful in diagnosis of CL in clinically suspected patients.

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Introduction

Leishmaniasis is a vector-borne parasitic disease caused by an obligate intracellular protozoan of the genus *Leishmania.* This disease is endemic in 66 Old World and 22 New World countries in the tropics, subtropics and southern Europe.[1,2,3,4] According to World Health Organization (WHO) estimates, 1.5 million cutaneous leishmaniasis cases and 500,000 visceral leishmaniasis cases occur annually and 12 million people were infected globally.[5] Thus, leishmaniasis is considered as an emerging and re-emerging infectious disease due to its wider geographical distribution with a high incidence.[5] Leishmaniasis is capable of causing a spectrum of clinical syndromes ranging from cutaneous ulcerations to systemic infections - cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL) or "kala-azar". In addition, fourth (diffused cutaneous leishmaniasis (DCL) and fifth forms (post-kala-azar dermal syndrome (PKDS) have also been described.[6,7]

The infective stage (promastigote) is transmitted to mammals via the bite of an infected female sandfly of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World.[8] Congenital and blood-borne transmissions have been reported.[9,10,11,12] Humans are generally considered to be an incidental host for leishmaniasis.[13]

In Sri Lanka, the first autochthonous leishmaniasis case was reported in 1992.[14] Since then, over 2500 CL cases have been reported islandwide.[15] Leishmaniasis was declared a notifiable disease by the Ministry of Health, Sri Lanka in 2006. Cutaneous leishmaniasis is the main form of the disease reported in Sri Lanka.[16] In addition, several VL[17] and atypical MCL[18] cases have been reported. *Leishmania donovani* (*L. donovani*) zymodeme MON-37 is responsible for CL in Sri Lanka[19] and the likely vector is *Phlebotomus argentipes* sibling species A.[20]

Diagnosis of CL in Sri Lanka is mainly based on direct microscopy. The occurrence of different morphometric forms of *L. donovani* amastigotes in CL patients has not yet been investigated in Sri Lanka. Therefore, the present study was carried out to identify the different morphometric forms of *L. donovani*.

Methods

Data and sample collection

This was a retrospective study. One hundred and thirty skin smears stored in the Department of Parasitology, Faculty of Medicine, University of Peradeniya were investigated to identify the different morphometric forms of *L. donovani*. In addition, demographic data (age, gender, occupation, household characteristics, and geographic area) were analyzed using department records. The presence of different forms of the amastigote was recorded.

Results

Demographic analysis

Hundred and thirty clinically suspected CL patients were referred from 8 districts (Kegalle, Kurunegala, Anuradhapura, Polonnaruwa, Puttalam, Jaffna and Matale) for diagnosis. All suspected patients were smear-positive for CL. Of them, 73 (56.15%) were males and 57 (43.85%) were females. Categorized by age, 56 (43.08%) patients were between 16-35 years and 53 (40.77%) between 36-60 years with 12 (9.23%) less than 15 years. The highest incidence of CL was reported from patients who were involved in outdoor activities (47.69%) followed by the unemployed group (44%). Patients confined to indoor activities had fewer infections (18.6%). Patients over 60 years were the least affected group (6.92%). Of the study population, 9.23% were living close to scrub jungles and 18.46% were associated with thatched or mud sheds. Domestic animals were present in the premises of 28 (21.54%) patients (Table 1).

Table 1: Demographics of CL patients

Demographics	No. of patients	Percentage (%)
Sex		
Male	73	56.15
Female	57	43.85
Age		
<15	12	9.23
16-35	56	43.08
36-60	53	40.77
>60	9	6.92
Occupation		
Outdoor	62	47.69
Indoor	24	18.46
Unemployed	44	33.85
Risk Factors		
Scrub jungle nearby	12	9.23
Ownership of domestic animals (cattle, goats etc.)	28	21.54
Thatched or mud sheds near by	24	18.46

Table 2: Distribution of lesions

Site of infection	No. of patients	Percentage (%)
Head (face and ears)	37	28.46
Neck	6	4.62
Upper limbs	46	35.38
Trunk	4	3.08
Lower limbs	24	18.46
Foot	8	6.15
Multiple sites	5	3.85

Distribution of skin lesions

Of the 130 patients, 35.38% had lesions on the upper limbs that were the most commonly affected body part. The second commonest place was the head (face and ears) (28.46%). Lesions on lower limbs were found in 18.6% of patients. The least affected area of the body was the trunk (3.08%). Four patients had lesions on the trunk and 3 of them were males while one was a female. Five patients (3.85%) had lesions on multiple sites and among them one patient had severe ulcerative lesions on the neck extending towards the trunk (Table 2).

Morphometrics of amastigote forms

All samples obtained from suspected CL patients (n=130) had amastigotes and/or promastigote-like structures (PLS). Amastigote forms alone were present in 65 (50%) patients and they were found in both intracellular and extracellular locations (Figures 1 and 2). Pseudocysts (a number of amastigotes in one cyst) can be clearly seen in Figure 1. The majority of patients (62) had extracellular amastigotes. Three patients had intracellular amastigotes while 19 patients had both

intracellular and extracellular located amastigotes. Amastigotes found in this study were either round (25.00%), oval (32.14%) or spindle (42.85%) in shape. The sizes of the amastigotes ranged from 2 µm to 5 µm (Figure 2).

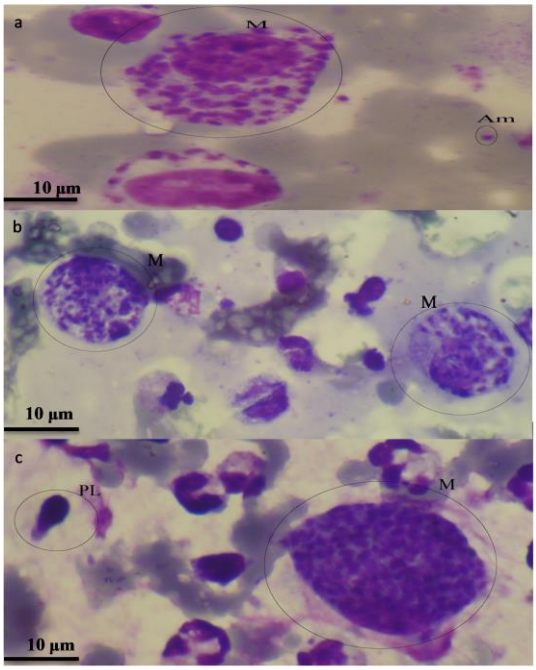

Figure 1: Skin smears stained with Giemsa (a, b and c).

Am - amastigote; M - pseudo cysts;
PL - promastigote-like structure.

Different morphometric features of the amastigotes that deviated from the typical shape (round or oval) are described for the first time in CL patient in Sri Lanka. This particular form was termed as "promastigote-like structures" (PLS). Promastigote-like structures were 6-35 µm in size (Figure 3). They all had a tail-like structure. Both PLS and amastigotes were seen in 19 (16.42%) patients. Promastigote-like structures alone were identified in 46 (35.38%) patients. Amastigotes alone were identified in patients who had lesions of less than two months duration. Patients with 2 to 8 month old lesions had both amastigotes and PLS. Over 9 months old lesions had only PLS (Table 3).

Table 3: Comparisons between duration of lesions and the presence of amastigotes and/or promastigotes-like (PLS) structures in CL patients

Duration (months)	Amastigotes alone (%)	Amastigotes+PLS (%)	PLS alone (%)
<2	43 (33.08)	-	-
3 to 5	19 (14.62)	7 (5.38)	-
6 to 8	3 (2.30)	12 (9.23)	-
9 to 12	-	-	38 (29.23)
>12	-	-	8 (6.15)

PLS; promastigote-like structure, n=130

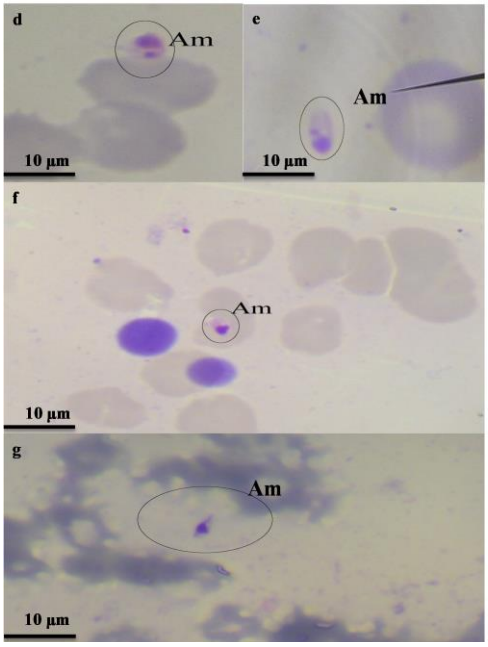

Figure 2: Different morphometrics of the amastigote.

Am – amastigote, d and f – round shaped, e – oval shaped, g – spindle shaped

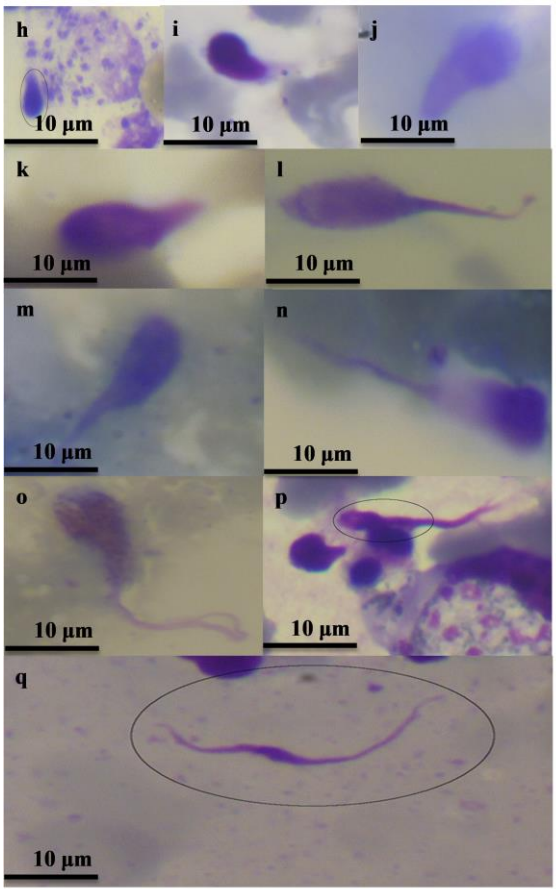

Figure 3: Transformation into promastigote-like structures.

h,i and j : condensed chromatin, cytoplasm disappears, grow larger and having a tail-like structure on one end.
k, l and m : candle flame shaped.
n, o and p : further growth of the tail-like structure.
q : transformation into fiber-forming stage (Daboul 2008)

Discussion

Demographic profile

Of the 130 CL cases, the majority were reported from Kandy (43.07%), Kegalle (17.69%), Matale (14.62%) and Kurunegala (13.08%) respectively. In this study, the spatial distribution of CL cases was expected to be biased since most of the patients referred to our laboratory were from Kandy, Kurunegala, Kegalle and Matale Hospitals. Nevertheless, previous studies and surveillance data have shown that the incidence of CL was much higher in the districts of Anuradhapura, Polonnaruwa, Hambanthota, Matara, Kurunegala, and Mullaitivu compared to that of other districts in Sri Lanka.[15,21,22,23,24]

According to the records, different cutaneous manifestations such as papules, nodules, and volcanic ulcers were present. Some CL patients had multiple lesions (29.23%). The number of lesions found in one patient ranged from 2 to 5 as reported by Nawaratna *et al.*, (2007) previously.[25]

It has been shown that uncovered parts of the body were more prone to sandfly bites than covered areas.[25,26,27] This may be the reason why lesions were often found in uncovered parts of the body. Male patients had a higher positivity rate (56.15%) than females (43.85%). This may be attributed to the increased outdoor activities, clothing preferences, occupation and other behavioral habits of these males. The duration of cutaneous lesions in CL patients were varied, 99.23% of whom had between weeks to 3 year old lesions. Only one patient had lesions of more than 6 years, which many have been due to treatment failures or reinfection.[25,28]

Cellular locations of amastigotes

Previous studies have shown amastigote forms of *Leishmania* species as an obligate intracellular protozoan.[29] However, contrary to previous investigations, research carried out by Daboul (2008) showed that *Leishmania* species do not only exist as an obligate intracellular parasite. Furthermore, the same study has shown the occurrence of amastigotes in both intracellular and extracellular locations.[30] In the present study as well, we have identified the amastigotes in both locations (Figures 1 and 2) in CL patients in Sri Lanka. Therefore, our study provides further evidence to prove that amastigotes exist in both intracellular and extracellular locations as shown by Daboul (2008).[30] It seems that amastigotes can survive in extracellular locations though the environment is not necessarily the same as in mononuclear cells. Based on those findings, the previous authors have speculated that *Leishmania* may no longer be considered as an obligate intracellular parasite, though more in depth investigations would be necessary to confirm such assumptions.[30]

Morphometrics of amastigote forms

We found different morphometric features of amastigotes (Figure 3) for the first time in CL patients in Sri Lanka. They were observed only in the extracellular locations. Daboul (2008) showed that the amastigote transforms into an ovum containing "promastigote embryo-like structure" once it comes out from the macrophage.[30] This ovum containing embryo-like structure is spindle-shaped, has a central nucleus surrounded by the cytoplasm (Figure 2, g). Daboul (2008) also suggested that the nucleus of amastigotes moves to one pole of the amastigote and the cytoplasm pushes the other pole in such a way leading to condensation of chromatin.[30] In the next stage of the development, the embryo grows larger in size and chromatin gets more condensed. Daboul (2008 and 2011) hypothesized this phenomenon as a "candle flame appearance" (Figure 3), while the cytoplasm is gradually reduced in size and disappeared.[30,31] The embryo continues to grow and form a tail-like structure. This tail-like structure develops further into flagellae and its appearance is quite similar to the promastigote (Figure 3). In addition, all other transformation stages that were described by Daboul (2008 and 2011) and Sharquie *et al.*,(2002) were found in our study as shown in Figures 1, 2 and 3.[30,32] Gradually, the flagella of promastigote-like structures get more condensed, thickened and enlarged up to 40 μm in length or more and Daboul (2008) described this process as transformation into a fibre–forming stage.[30] Sharquie *et al.*,(2002) have shown the presence of a fibre forming stage in the dry nodular type lesion.[32] This process may lead to an inflammatory fibre granulomatous immune reaction associated with lymphocytes, mononuclear cells, and a variable number of plasma cells.[30,32] At the end of this process, fibres are elongated and thickened from both sides, while the nucleus is condensed into the middle, getting thinner and smaller (Figure 3, p-q). At this point, a few plasma cells and lymphocytes can be seen, and the lesion is dry and about to heal leaving a permanent scar.[30] Daboul (2008) has suggested that the post healing lifetime scar may be due to pseudo-fibres of the parasite itself and not as a result of the human fibroblasts.[30]

In Sri Lanka, diagnosis of CL has been carried out routinely using clinical symptoms along with direct microscopy with the presence of amastigotes confirming the diagnosis and absence of same excluding CL. However, the sensitivity of the direct microscopic method is only 60-70%.[33] Therefore over 30% - 40% of cases may not be diagnosed by direct microscopy. The low sensitivity of the direct microscopic method may be due to the progression of the disease and low parasite counts in the lesion.[34] According to a recent study, amastigotes may also not be found in clinically hyperactive and inflamed CL lesions.[35] Also, it further suggested that amastigotes could be able to survive in the extracellular fluid. Environmental conditions in extracellular fluid are harsher than within macrophages. In order to survive in such conditions, amastigotes might have transformed into promastigote-like forms**.** As Daboul (2013) has described, promastigote-like structures become active and penetrate the subcutaneous tissues and cause the real signs and symptoms of the disease at this stage.[35] The morphometric features of promastigote–like structures and candle flame form are active forms of amastigotes that are transformed into fibres gradually. Similar morphometric features were identified in CL patients in Sri Lanka. Amastigotes were frequently found in patients who had less than 5 month old lesions. This finding suggests that amastigote forms are more common at the early stages of disease progression and promastigote-like structures can be found in later stages (Table 3).

Conclusions

Microscopic examination of skin smears showed the presence of promastigote-like structures for the first time in CL patients in Sri Lanka. Amastigotes were predominantly present at the early stages of cutaneous lesions while promastigote-like structures were more commonly seen in older lesions. Therefore, we suggest that different morphometric features of amastigotes should not be ignored as they may be useful in the diagnosis of CL in clinically suspected patients.

Limitations of the study

Identification of different forms of the amastigotes was done using the light microscope only. The use of a transmission electron microscope or other molecular techniques such as DNA analysis would have been useful to identify the different forms of these amastigotes.

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