Original Article

INFECTIOUS Microbes & Diseases

OPEN

Cytomorphological Variations of Leishman-Donovan Bodies Found in Cutaneous Leishmaniasis Patients in Sri Lanka

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Abstract

Leishmania parasites undergo a dimorphic life cycle. Diagnosis of cutaneous leishmaniasis (CL) is primarily made by the microscopic identification of the amastigote form of the parasite in a smear. Therefore, correct identification of Leishman-Donovan (LD) bodies in smears is crucial for CL confirmation. The aim of this study was to provide a broad overview of different cytomorphologies of LD bodies to improve the sensitivity of microscopic detection of *Leishmania donovani*, which causes CL in Sri Lanka. A total of 125 smears, prepared from patients who met the positive diagnostic criteria of CL, were Giemsa stained and examined microscopically. The density, cellular location and all possible cytomorphological forms of the parasite were documented. A total of 10 different cytomorphological forms of LD bodies were identified. Among them, the round form was observed only intracellularly. The other morphological forms, namely the pear shape, cigarette shape, candle flame form, embryo shape, flagellate form, binary form and rosette form, were found only extracellularly. The spindle form and lens shape were observed in both intracellular and extracellular locations. *L. donovani* amastigotes have a wide range of morphologies besides their classical forms. Although *Leishmania* are considered as obligatory intracellular parasites, they manage to survive successfully within the extracellular hostile conditions as well. Therefore, having a broader view of different morphological forms for *Leishmania* parasites may help to improve diagnosis of CL.

Keywords: cutaneous leishmaniasis; *Leishmania donovani*; cytomorphology; amastigote; diagnostic; microscopy

Introduction

Leishmania, one of the obligate intracellular kinetoplastid protozoan parasites, causes a variety of disease states in humans and other mammals, which include a broad spectrum of disease manifestations, such as local self-healing cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis and invariably fatal visceral leishmaniasis.[1–3] Currently, leishmaniasis has received a renewed interest due to the large scale emergence and reemergence of leishmaniasis cases.[4] One of the most dramatic examples is the new focus on CL in Sri Lanka, which is caused by a genetically distinct variant of *Leishmania donovani* zymodeme MON-37. *Phlebotomus argentipes*, the vector of *L. donovani*, is a widely prevalent insect in almost all parts of Sri Lanka.[4–11] The first case of locally acquired leishmaniasis was documented in 1992[12] and the clinical features of the initial patients in this epidemic were reported in 2003, confirming the presence of locally acquired CL in Sri Lanka.[7]

Leishmania parasites have a dimorphic life cycle as either a motile promastigote flagellar form or a nonmotile amastigote form. The infective extracellular promastigotes are found in the midgut of female vectors of certain species of sandflies of the genus *Phlebotomus* (old world) and *Lutzomyia* (new world).[1,13] The amastigote form, which lacks an exteriorized flagellum, has been driven to parasitize within the dermis of the vertebrate host's mononuclear phagocytic cells and reside within acidified parasitophorous vacuoles.[14] In the first peripheral site, *Leishmania* parasites initiate complex processes forming a microenvironment that favors their setting in and their ultimate fate. These complex processes involve both innate and adaptive immune mechanisms.[15–18] Within the sand fly vector, the amastigotes, acquired through infected mammals, transform into distinct stages from procyclic promastigotes to mammal infective metacyclic promastigotes.[19,20] According to the blocked fly

Editor: Stijn van der Veen

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Author contributions: GWR and AM designed the study; RA provided samples and clinical data; GWR, KPA and AM prepared the manuscript; KPA analyzed and interpreted the data; all authors have equally accepted responsibility for the entire content of this manuscript and approved its submission.

Ethics: Research involving human subjects complied with all relevant national regulations and institutional policies, and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013) and has been approved by the ethics review committee of Faculty of Medicine, University of Jaffna (Ref No.: J/ERC/15/68/NDR/0136), which was approved on December 17, 2015. Written informed consent was obtained from all individuals included in this study.

Data availability: All data generated or analyzed during this study are included in this published article. Additional project information for the current study can be obtained from the corresponding author on reasonable request.

Funding: No funding was received for this work.

Conflicts of interest: The authors reported no conflicts of interest.

Infectious Microbes & Diseases (2023) 5:2

Received: 31 December 2022/Accepted: 10 April 2023/First published online: 2 May 2023

http://dx.doi.org/10.1097/IM9.0000000000000118

hypothesis, the metacyclic promastigotes are able to form a physical obstruction in the vector that has to be removed by regurgitation during natural transmission.[1,19,21] After the successive transmission, the amastigote-form parasites initially do not prefer to present in the peripheral circulation, but instead they present in the infected mammalian host's skin, with a vast array of clinical manifestations.[1,22]

CL occurs at the site of the skin where *L. donovani* parasites have been regurgitated by infected sand flies,[1] and it initially can be observed as a reddish, inflamed swelling or lump. Over the subsequent months, the swelling grows until the skin ultimately opens in the form of an ulcer with a raised border and a crusty center.[23,24] Incubation periods can vary from a few weeks to many months; hence, the ulcer develops into distinct types of wet and dry lesions, such as nodules, plaques, ulcerative skin or open lesions, after the establishment of the infection.[25] These manifestations of lesions can be either single or multiple, leading to disease relapses based on the durability, size of the lesion, host immunity, density of parasites and other undefined factors. Sand flies generally bite hosts on exposed skin areas, for example, face and hands.

CL has broad differential diagnostic methods, which are important because diseases with other causative agents that are common in leishmaniasis endemic areas have similar clinical symptoms as leishmaniasis, such as leprosy, skin cancers, tuberculosis and cutaneous mycoses.[26] However, microscopic examination of Giemsa-stained biopsy smears or aspirates is the primary and conventional gold standard for diagnosing CL.[26] However, leishmaniasis can be a challenging disease to diagnose, especially when no clear amastigotes are observable due to the absence of its typical morphology in Giemsa-stained biopsy smears. Unusual morphological appearances may cause uncertainty to achieve an accurate diagnosis and may result in a false-negative diagnosis.

The present study was designed to investigate all possible morphological aspects of *Leishmania* parasites; microscopic smears were employed to identify the various exceptional morphological shapes of Leishman-Donovan (LD) amastigote bodies within human cutaneous lesions. However, the uttermost purpose of the study was to improve the sensitivity of the traditional microscopic smear examination technique and provide an extensive overview of the different cytomorphologies of LD bodies.

Results

Among 145 suspected CL patients, 125 patients (86.21%) finally met the inclusion criteria for positive CL diagnosis both by microscopic identification of any kind of cytomorphological form of *Leishmania* parasites and polymerase chain reaction (PCR) detection of *Leishmania*-specific kinetoplastic minicircle DNA. Although PCR showed a high sensitivity by detecting 134 positive patients (92.40%), 9 PCR-positive patients (6.20%) were not positive by microscopic examination.

Findings of standardized graded direct microscopy

Standardized smear grading was performed for the 125 patients who met the diagnostic criteria for CL and were confirmed through microscopic smear examination. The density of parasites within the smears varied considerably. Grades of 2+ (27.2%) and 3+ (23.2%) were found most commonly, while 12% of the smears were graded as 1+, 16.8% as 4+, 13.6% as 5+, and only 7.2% contained the highest density of amastigotes and were graded as 6+.

With respect to the location of amastigotes, amastigotes were found only extracellular (Figure 1A) in slides of 52 cases (41.6%), while 26 slides (20.8%) displayed only intracellular amastigotes (Figure 1B). Simultaneous presence of both intracellular and extracellular amastigotes (Figure 1C) was observed in 47 smears (37.6%). Among the 125 positive smears, only 8 smears (6.40%) were found to have the typical amastigote form alone. There were 90 slides (72.00%) that presented both typical and atypical amastigote forms in the same smear. Altogether, we encountered 27 slides (21.60%) displaying only the atypical amastigote forms. Among those, 10 (37.04%) were found with one atypical amastigote form only and there were 17 slides (62.96%) that had either two or more atypical cytomorphological forms. None of the slides displayed all possible cytomorphological forms within the same smear.

Cytomorphology of different amastigotes forms

Typical amastigote morphologies, consisting of a round or oval shape with separated nucleus and kinetoplast (Figure 1D), were mostly expected. However, the morphological data demonstrated that *Leishmania* amastigotes on Giemsa-stained smears varied noticeably, displaying irregular and unfamiliar polymorphic cytomorphologies. The most interesting finding was the presence of promastigote-like forms with flagellar structure (Figure 1E), which has previously been documented by Daboul.[27] Routine examinations and overall assessments revealed that the irregular forms of *L. donovani* amastigotes showed various changes in the development of their cell structures, which prompted an analysis of all possible cytomorphologies within the human host (Table 1).

Round or oval form. So far, it has been adopted that the conventional morphology of the *Leishmania* amastigote form is a round or oval shape, with a central round nucleus and a small rod-shaped kinetoplast in the vicinity of the nucleus. We found total of 68 smears with this classical morphology (Figure 2A and B).

Spindle form. The spindle form is tapered at each end, which is also referred as fusiform as two ends are pointed. As Daboul[27] reported earlier, the nucleus starts to take a polarized position inside the amastigote and the cytoplasm appears on one side of the cell. In addition, chromatin can be seen in a condensed form. According to our study, the spindle shape is the most common recorded extracellular irregular form of amastigotes, which was observed in 63 smears (Figure 2C and D).

Pear shape. The pear shape of the amastigote becomes tapering toward the anterior end of the cell, whereas the base is broadened due to the accumulation of cytoplasm at the posterior end. The position of the nucleus can be seen either in a polarized position or at a side of the cytoplasm. The kinetoplast cannot be distinguished from the nucleus owing to condensation of the chromatin found within the nucleus (Figure 2E and F).

Lens shape. The lens shape was observed in 28 smears and displays a broadening central portion with a condensed nucleus (Figure 2G and H).

Cigarette shape. Amastigotes are observed in a cigarette shape with a long and cylindrical structure. Cells appear with blunted ends and centralized nucleus (Figure 2I and J).

Candle flame form. As a gradual consequence of nucleus enlargement, the candle flame form appears due to a decrease in size of amastigote cytoplasm, and a tiny tail-like forwarded projection can be seen in smears examined. They look like a narrow promastigote body without flagella (Figure 2K and L).

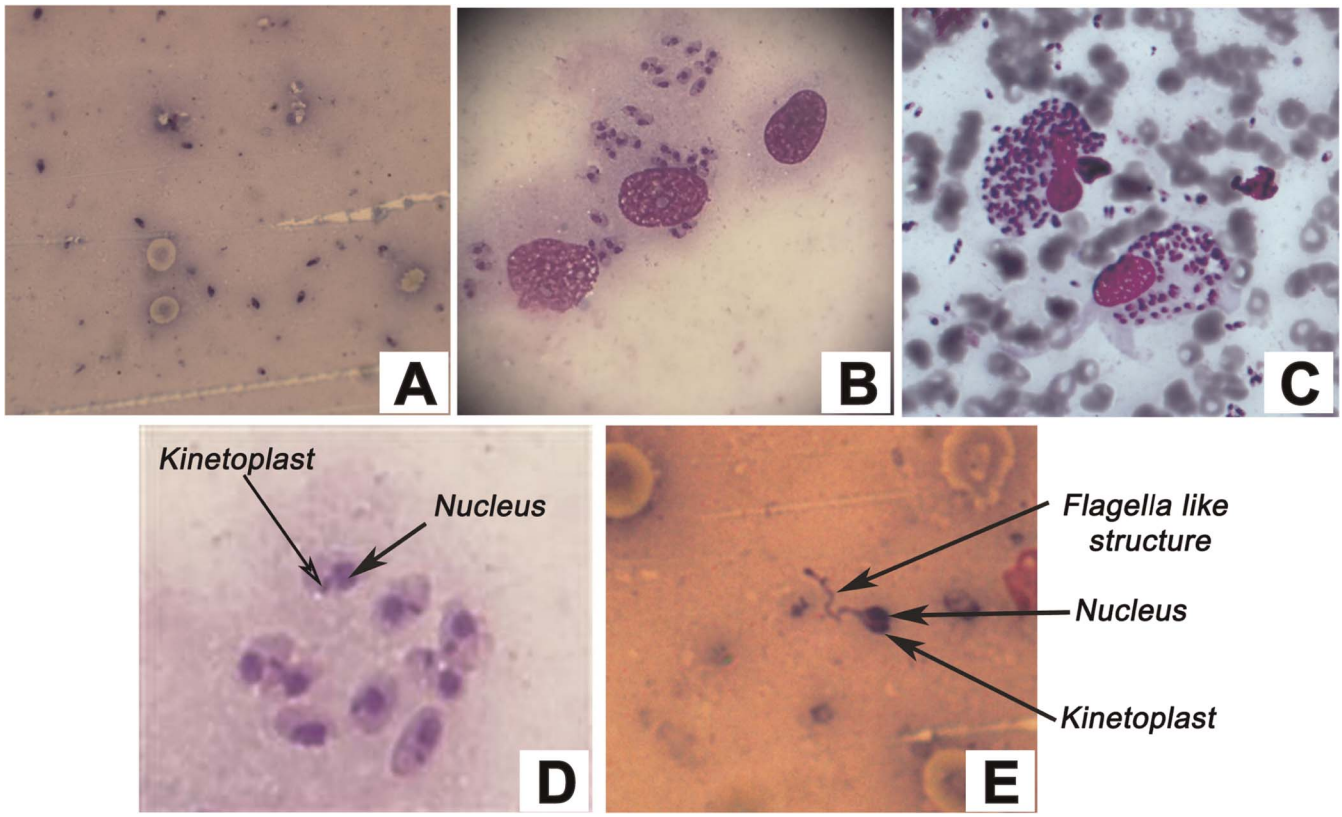

Figure 1. Different locations of amastigotes. A: Amastigotes in the extracellular environment. B: Amastigotes in the intracellular environment. C: Amastigotes in both the intracellular and extracellular environments. D: Typical amastigote morphologies. E: A paramastigote.

Flagellate shape. This morphology mimics the cytomorphology of the procyclic promastigote state within the sand fly vector. Here, the tail-like projection is protruding outside and presented as flagella-like structures of varying sizes. Altogether, 27 slides were found with this amastigote shape as long flagellates (Figure 2M, N and O) and short flagellates (Figure 2P, Q and R).

Embryo shape. As a result of continuous growth of the candle flame shape, the posterior blunted end becomes broadened while the anterior end narrows, which can be observed as a small handle protruding outside (Figure 2S and T). This is similar to the shape of a paramecium in some cases.

Binary form. The appearance of this morphology may be seen as if two individuals are attached together. Since *Leishmania* is an obligatory intracellular parasite, its propagation by means of binary fission is necessarily attained in an intracellular environment. Thus, the manifestation as an attached form can be possibly due to incomplete separation (Figure 2U and V).

Rosette form. Rosette form appears as a circular cluster of amastigotes, which is associated with the extracellular environment. This form is rarely observed and only appeared in limited number of slides (Figure 2W and X).

Table 1

Different morphologies of amastigotes and their cellular locations in laboratory-confirmed cutaneous leishmaniasis-positive smears

Morphology	No. positive smears[a]	Location		Percentage (%)[b]
		Intracellular	Extracellular	
Round/oval	68	✓		23.05
Spindle	63	✓	✓	21.36
Pear	34		✓	11.53
Lens	28	✓	✓	9.49
Cigarette	24		✓	8.14
Candle flame	22		✓	7.45
Embryo	11		✓	3.73
Flagellate	27		✓	9.15
Binary	10		✓	3.39
Rosette	8		✓	2.71

[a]Based on 125 patients.

[b]Based on 295 total morphological observations.

Associations between cytomorphological types and smear grading

Logistic regression analysis between amastigote morphological types and smear grading revealed that the classical round/oval morphological form was more likely to be present in smears with high parasite densities (in grades 3+, 4+, 5+ and 6+). Spindle and rosette shapes were found to be more significant for grade 5+ smears, and lens and embryo shapes for grade 2+ and 6+ smears, respectively. A significant association was observed between flagellate shape and smears with low parasite densities (in grades 1+ and 2+). The *P* value and marginal effect values obtained from regression analysis are given in Table 2. Besides standard intracellular amastigote forms, the presence of polymorphic amastigote forms both in the

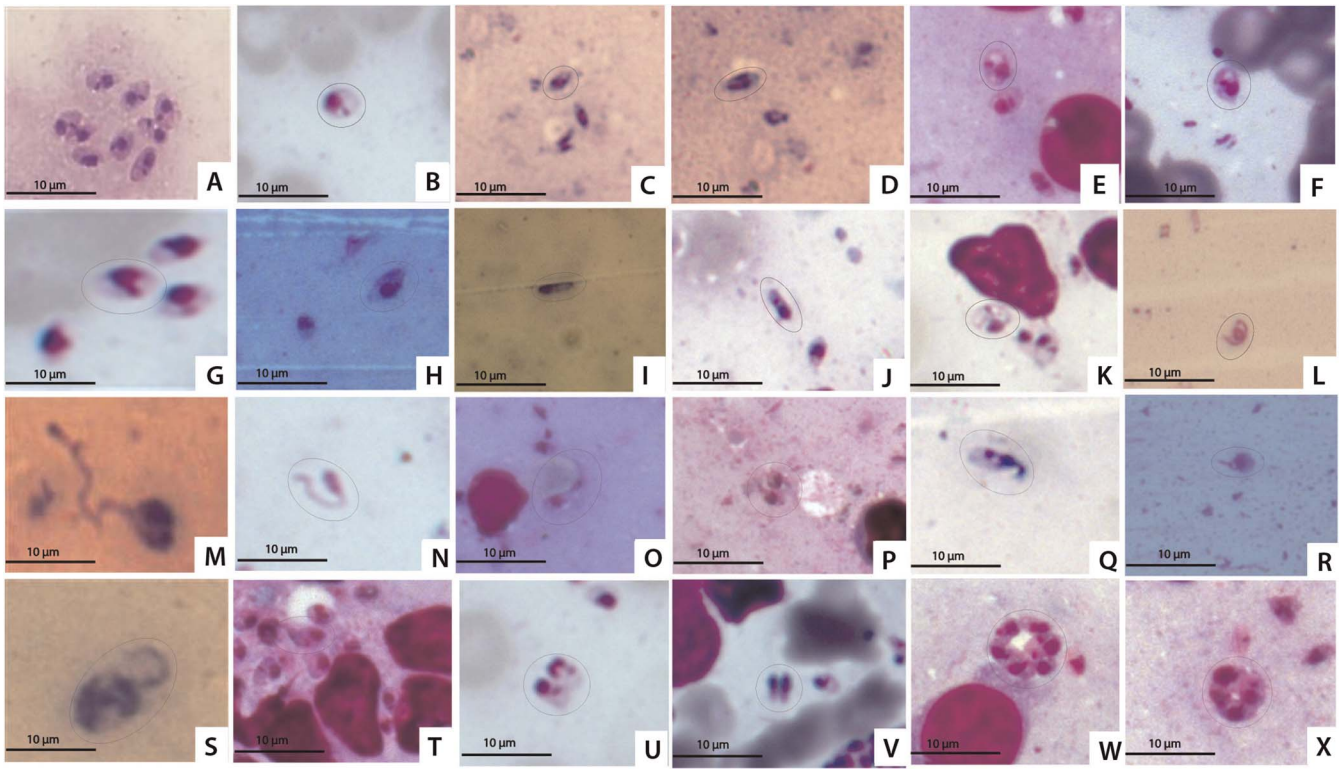

Figure 2. Different morphologies and shapes for Leishman-Donovan bodies. A: Oval form. B: Round form. C and D: Spindle form. E and F: Pear shape. G and H: Lens shape. I and J: Cigarette shape. K and L: Candle flame form. M, N and O: Long flagellate form. P, Q and R: Short flagellate form. S and T: Embryo shape. U and V: Binary form. W and X: Rosette form.

intracellular and extracellular environment suggested the possibility for a morphological transformation of amastigote forms, which may contribute to the sustenance of subsequent generations of *L. donovani* amastigotes within the definitive human host (Figure 3).

Discussion

L. donovani is the etiological agent of visceral leishmaniasis in most leishmaniasis endemic areas in the world,[28] although the same species acts as the causative parasite for CL in some countries,[29] including Sri Lanka.[7] Distinct morphological events of *L. donovani* are involved within the vector *P. argentipes*, which has been investigated to provide a detailed description of the parasitic promastigote life cycle.[30–33] Conversely, the structural components and morphological changes for amastigotes, the interplay between the proliferative cell cycle and differentiation to the next life cycle stage, and *Leishmania* mammalian host interaction are rarely known.[34]

Usually, for metacyclic promastigotes to be inoculated by a sand fly bite, they are preadapted to the transformation strategy into amastigotes.[1] Probably the same phenomena can happen vice versa within lesions up to some extent. During the first inoculation, metacyclic promastigotes, which transform into amastigotes immediately, should start their survival cycle in order to develop in the procyclic stage. It is crucial to emphasize that the aforementioned drastic morphological changes of the *Leishmania* parasite during its amastigote life cycle are unable to be achieved completely by most individual *Leishmania* parasites. Indeed, very few can continue their transformation until the end and remain for a long period of time within the ulcer to cause pathogenicity. Moreover, amastigotes are the disseminating form in mammals and it is commonly assumed that after their release from infected macrophages, they can be phagocytized by adjacent macrophages. The amastigotes that have less capacity to reach at least the initial stages of transformation before being released by the macrophages will be destroyed within a limited period of time in which the host immune system exerts its actions. However, host immune competence determines the level of susceptibility or resistance to further infection.[35]

Cytodiagnosis techniques, such as microscopic smear examination and in vitro culturing of parasites, have been considered as the

Table 2

Association between different *Leishmania donovani* amastigote cytomorphological types/forms and smear grading

Morphology	Smear grading	*P*	Marginal effect
Round or oval	**3+**	<0.001	0.15
	4+	<0.001	0.25
	5+	<0.001	0.17
	6+	0.011	0.06
Spindle	**5+**	0.005	0.16
Lens	**2+**	0.013	0.25
Embryo	**6+**	0.012	0.045
Flagellate	**1+**	0.012	0.23
	2+	<0.001	0.41
Rosette	**5+**	0.023	0.41

The data in boldface format correspond to the grading of amastigote numbers observed in Giemsa stain smears, which is given in Table 3.

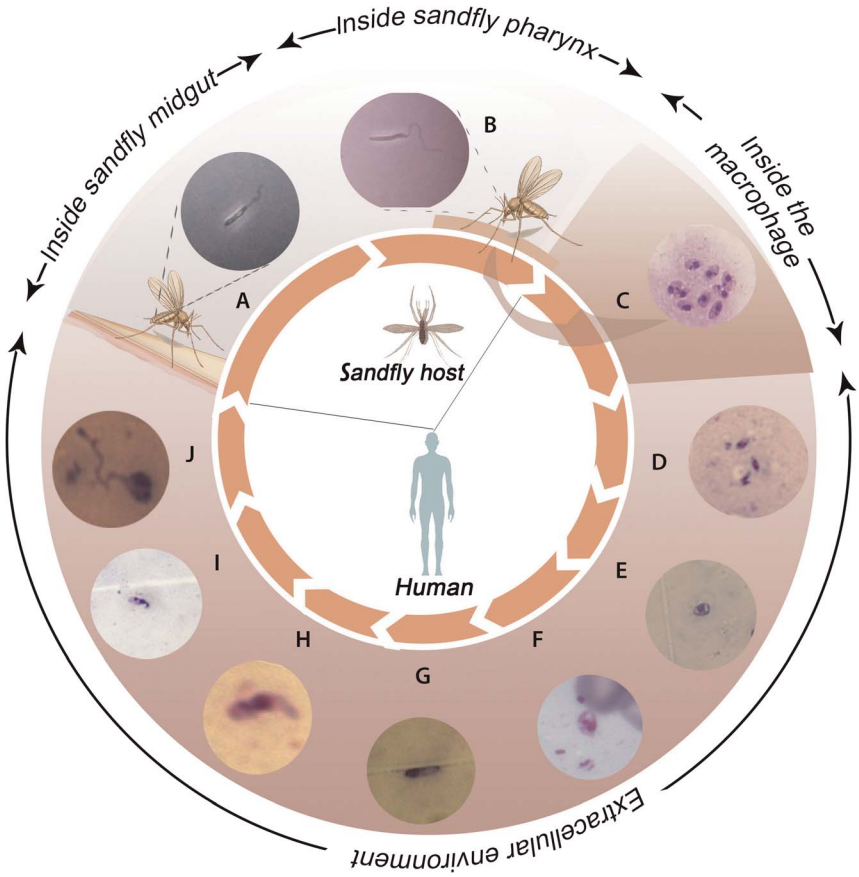

Figure 3. Intracellular and extracellular polymorphic stages in the life cycle of *Leishmania donovani.* A: Procyclic promsastigotes. B: Metacyclic promastigotes. C: Classical oval- and round-form amastigotes. D: Spindle-form amastigotes. E: Pear-shape amastigotes. F: Lens-shape amastigotes. G: Cigarette-shape amastigotes. H: Candle flame-form amastigotes. I: Short flagellates. J: Paramastigotes.

primary parasitological diagnosis methods, which can be applied to confirm CL by visualization of parasites. Despite challenges in detecting parasites in the smear, microscopic smear examination is still preferred as the gold standard for confirmation of CL.[36] Although microscopic examination is relatively high in specificity, the sensitivity is low compared to a standard PCR,[37] as was also observed in our study by detecting a total of 134 patients by PCR among the 145 clinically suspected CL patients. However, only 125 patients were detected through the microscopic method. Lower sensitivity of the microscopic examination method can be associated with the duration between sample collection and microscopic observation, which may lower the amastigotes loads,[38] the poor sample collection method and the possible transformation mechanisms of the amastigotes.

Besides these adverse factors, routine examination of smears is able to detect a massive number of erratic, divergent and unfamiliar cytomorphologies, which deviate from classical appearances (round or oval) of LD bodies. Although these parasites are considered obligatory intracellular parasites, our observations revealed that they colonize both intracellular and extracellular environments, and interestingly, the bulk of them were in extracellular sites and only limited numbers in intracellular spaces. The correlation observed between their morphologies and the locations indicates that almost all irregular forms are present in the extracellular hostile environment, while the regular classical forms are trapped within their host cells. Hence, there should be a possible safeguard mechanism for their survival after the discharging of parasites into the immediate vicinity of the macrophages and other immune cells.

As far as the morphological assay, the classical morphology of a LD body is comprised of the nonflagellated round or oval shaped body. The nucleus and kinetoplast can be seen separately while there is no such separated kinetoplast in the majority of the irregular LD bodies. In addition to the regular amastigote forms, *L. donovani* amastigotes contain a number of different morphometric shapes with no defined functional distinction. Therefore, having an idea about all possible amastigote cytomorphologies is crucial for the precise identification of the parasite. If not, it is comprehensible only to experts of the field.

To our knowledge, very few studies have been performed previously that identified morphological features of the *Leishmania* amastigote form. Overall assessment of all possible morphological forms identified from our study reveals that the spindle shape, which tapers at either ends with a polarized nucleus, was the predominant irregular morphological form (in 63 smears). This is in agreement with similar findings reported by other researchers.[39–41] The least common morphological form was the rosette form, this circular cluster of amastigotes was identified in eight slides, and the same form has been reported previously.[42] The pear shape was the second most common form in 34 smears in our study, followed by lens shape in 28 smears, cigarette shape in 24 smears and candle

flame form in 22 smears. The rest of the morphologies included embryo shape in 11 cases and binary form in 10 smears. Except the lens-shaped LD bodies, most of the above irregular morphological forms were also identified from other studies conducted in Middle East countries where *Leishmania major* causes CL.[39,41,42]

Interestingly, among the polymorphic variations, a limited number of amastigotes with an external small flagellum was observed during our survey. According to Daboul,[43] these features can be explained as a type of promastigote (procyclic promastigote stage) and this unique figure of LD bodies can be named as "paramastigote" (Figure 1E). Spotin et al.[42] also identified the similar structures with a small flagellum and reported it as an exceptional morphological form of amastigotes. It has been reported in previous studies that the parasites were often phagocytosed with the cell body entering the macrophages first (anterior region) and if they were about to interact with the macrophages via their flagellum, they would rapidly turn around and finally enter into the macrophages by the cell body.[44] The similar structures identified in our study also suggest that it might be an intermediate stage present in the extracellular environment and this flagella-like structure may play an important role in infecting new macrophages. It is worth noting that the "paramastigote" form mimics some of the characteristic features of a true promastigote with relatively limited physiological activities. Presence of both regular and irregular cytomorphological forms in the extracellular environment in relation to their transformation strategy suggests the possibility for some kind of adaptive switch mechanism to ensure their survival after release in the extracellular space, while creating a pathway to be phagocytosed and replicated inside the macrophages (the definitive host cells). These assumptions can be correlated with the recent data from French Guiana, suggesting that even in moderately immunosuppressed HIV-infected individuals, CL is characterized by a higher rate of recurrence and is more difficult to treat than for HIV-negative individuals.[45]

Based on the analysis of Giemsa-stained smears, it has been confirmed that *L. donovani* amastigotes are the disseminating form of CL and have a greater polymorphism than their classical forms. Although they are considered as obligatory intracellular parasites, they successfully survived within the extracellular hostile conditions. While the classical forms of LD bodies are restricted within the intracellular vacuoles, massive number of irregular forms inhabit in the extracellular environment. These different extracellular polymorphic forms are observed in smears, but mostly ignored or considered as artifacts during the microscopic examination. In addition, the results of logistic regression show that the classical forms of amastigotes have a significant association with smears that have a considerably high parasite density, while the spindle and flagellate forms show a significant association with smears with low parasite densities. This suggests that there is a possibility to notice the unusual morphological forms (spindle and flagellate) among the smears with low parasite densities during screening of smears under the microscope. Hence, it is valuable to be aware of all the possible morphological changes in LD bodies presenting in both intracellular and extracellular environments in terms of increasing the sensitivity of microscopic diagnosis for CL.

Materials and methods

Samples

A total of 145 clinically suspected CL patients were recruited for this study based on the characteristic skin lesions and the suggestive clinical history. Included patients presented to the skin clinic at District GH Vavuniya between December 2015 and December 2016. All the patients were inhabitants of different parts of the North Central Province, Sri Lanka. Prior to commencement of the treatment, slit skin smear samples obtained from the suspected patients were subjected to direct microscopic analysis to confirm the presence of any kind of cytomorphological form of *Leishmania* parasites, followed by PCR amplification of *Leishmania* specific kinetoplastic minicircle DNA to further confirm the true positivity of samples. Confirmation of CL patients both by microscopic identification and PCR was considered as the sample inclusion criteria for this study.

Our study was carried out with the approval of the ethics review committee of Faculty of Medicine of University of Jaffna (Ref No.: J/ERC/15/68/NDR/0136), which was approved on December 17, 2015. Written informed consent was obtained from all individuals included in this study.

Microscopic examination

All the smears were stained following the modified Giemsa staining method.[46] Briefly, 10% Giemsa stain was prepared using 10 mL of Giemsa stain solution (Sigma-Aldrich) in 90 mL of phosphate buffer (pH 7.2). A fresh stain was prepared every third day and filtered just before use.

The aspirated cytology material directly obtained from the lesions by the dermatologist was spread on a clean and alcohol-free microscope slide, and then covered by another one. Both thin and thick smears were prepared for the microscopic and PCR analysis, respectively. The prepared smears on the glass slides were air-dried for 2–3 minutes and sent to the laboratory. The air-dried thin smears were fixed by briefly dipping into methanol in a Coplin jar 3 times for 20–30 seconds. After fixation, the slides were immediately placed in the filtered 10% Giemsa stain solution in a staining jar for 10 minutes with gentle agitation. The slides were then washed by three gentle dips in phosphate buffer (pH 7.2) and placed upright to air-dry.

Altogether, 125 Giemsa-stained slit smears from confirmed positive CL cases through microscopic diagnosis were used to identify the morphological variations. The thin smears were examined under a light microscope (Zeiss AxioscopeA1) with a plan-neofluar 100×/1.25 oil immersion to identify different cytomorphologies of LD bodies, which repeatedly appeared in different lesions. The amastigote densities of all positive smears were graded from 1+ to 6+.[47] All amastigote polymorphisms and their locations (intracellular or extracellular) were documented. Images of all amastigote polymorphisms were captured at 1000× magnification with a Zeiss AxioscopeA1 microscope equipped with AxioCam MRc5 CCD camera. The scale bar was 10 μm.

Grading of slides. The positive smears were graded for the presence of amastigote numbers based on World Health Organization guidelines (Table 3).[47]

Molecular confirmation

Precise identification of all the smears was confirmed by PCR using *Leishmania* species–specific primers in order to confirm the true positivity and also to exclude the possibilities of identifying any artifacts as LD bodies.

DNA extraction. After the microscopic identification, DNA was extracted from all the thick smears by scraping the smear surface with a scalpel blade, which was put into 1.5-mL Eppendorf tubes containing 300 μL of nuclease free water. The samples were mixed by vortexing until the materials were completely dissolved and boiled for 10 minutes. The samples were subjected to DNA extraction by

Table 3

Grading of amastigote numbers according to World Health Organization guidelines

Grade	Average parasite density[a]
6+	>100 parasites/field
5+	10–100 parasites/field
4+	1–10 parasites/field
3+	1–10 parasites/10 fields
2+	1–10 parasites/100 fields
1+	1–10 parasites/1000 fields
0	0 parasite/1000 fields

[a]Using 10× eyepieces and 100× oil-immersion lens.

using the DNeasy Blood and Tissue Kit (QIAGEN GmbH) and stored at –20°C.

PCR detection. *Leishmania* parasites were identified by kinetoplast DNA using the primers [F: 5′-GGCCCACTATATTACACCAACCCC-3′ and R: 5′-GGGGTAGGGGCGTTCTGCGAA-3′] to amplify a 120-bp length sequence from the conserved region of kinetoplastic minicircle DNA from *Leishmania* spp.[48] PCR was performed in a 50-µL reaction consisting of 1.5 mM $MgCl_2$, 200 µM dNTPs mix, 50 ng of each primer, 1 U of Taq polymerase (Promega) and 50–75 ng of extracted DNA. The PCR run was programmed with an initial denaturation step at 94°C for 6 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR products were analyzed by electrophoresis on 1% agarose gels in Tris-acetate EDTA (TAE) buffer. DNA isolated from the cultured parasite was also subjected to PCR experiments as positive controls.

Statistical analysis

All the verified data were compiled in to a MS excel sheet. Statistical analysis was carried out using R version 4.2.1. The significant morphological forms of amastigotes associated with the grading of smears were analyzed using multinomial logistic regression, following the backward elimination method. Smear grading was considered as the dependent variable and cytomorphological forms were treated as the independent variables. "Grade 0" was considered as the base level. $P < 0.05$ was considered as the level of significance. The "multinom" function from the "nnet" package was used to estimate the multinomial logistic regression values. In addition, the "marginaleffects" function from the "marginaleffects" package was used to estimate the average marginal effects.

Acknowledgments

The authors thank the staff attached to the Dermatology Clinic, District General Hospital Vavuniya, for giving their tremendous support. The authors also thank all the technical staff members of Department of Parasitology, Faculty of Medicine, University of Jaffna.

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How to cite this article: Murugananthan A, Amarasinghe KP, Rathnaweera GW, Amalraajan PT, Arulenthiran R. Cytomorphological variations of Leishman-Donovan bodies found in cutaneous leishmaniasis patients in Sri Lanka. *Infect Microb Dis* 2023;5(2):93–100. doi: 10.1097/IM9.0000000000000118