Chronic generalized fibrotic skin lesions from disseminated leishmaniasis caused by *Leishmania martiniquensis* in two patients from northern Thailand infected with HIV

S. Chiewchanvit,¹ N. Tovanabutra,¹ N. Jariyapan,² M.D. Bates,³ P. Mahanupab,⁴ M. Chuamanochan,¹ A. Tantiworawit¹ and P.A. Bates³

Departments of ¹Internal Medicine, ²Parasitology and ³Pathology, Chiang Mai University, Chiang Mai 50200, Thailand
³Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster LA1 4YG, U.K.

**Summary**

Background Leishmaniasis is a newly emerging infection in Thailand. Most of the previous human cases have presented with the clinical features of visceral leishmaniasis and were mainly found in southern Thailand. Here we report the first two patients from northern Thailand presenting with disseminated cutaneous leishmaniasis.

Objectives To determine the nature of the infection of leishmaniasis and to identify the species of parasite responsible.

Methods Clinical investigations included the taking of biopsy samples and histology. Parasitological diagnosis was performed by establishment of *Leishmania* promastigote cultures, and identification was performed by DNA sequencing of four independent gene loci (ribosomal RNA internal transcribed spacer 1; large subunit of RNA polymerase II; heat shock protein 70; RPL23a intergenic sequence).

Results Both patients were infected with HIV, and had multiple cutaneous lesions and accompanying visceral leishmaniasis. They had similar cutaneous manifestations characterized by chronic generalized fibrotic lesions, which were more prominent on traumatic areas. In both patients the parasite was identified as *Leishmania martiniquensis*. This was a recently described species that is distinct and only distantly related to the classical agents of cutaneous leishmaniasis in Asia (*Leishmania major* and *Leishmania tropica*) or of visceral leishmaniasis (*Leishmania donovani* and *Leishmania infantum*). Each patient responded well to therapy with intravenous amphotericin B followed by oral itraconazole.

Conclusions *Leishmania martiniquensis* is a cause of cutaneous leishmaniasis in Thailand.

What’s already known about this topic?
- Leishmaniasis is an emerging disease in Thailand, presenting as cutaneous or visceral leishmaniasis, sometimes with accompanying HIV infection.
- Most of the reported infections have been attributed to so-called ‘*Leishmania siamensis*’, but this identification has been called into question.

What does this study add?
- Here we demonstrate that cutaneous leishmaniasis in two patients from northern Thailand is caused by *Leishmania martiniquensis*.
- Retrospective analysis indicates that the majority of cutaneous leishmaniasis in Thailand is caused by *L. martiniquensis*.
Leishmaniasis is a group of infectious parasitic diseases caused by various species of the protozoan genus *Leishmania*, transmitted by the bites of female phlebotomine sandflies.\(^1\) The clinical presentation of leishmaniasis can vary, depending on the infecting species and the response of the human host; however, the clinical syndromes of leishmaniasis can be divided into three main forms: visceral, cutaneous and mucocutaneous.\(^2\) Mortality is mainly associated with visceral infection, while the cutaneous forms usually only cause morbidity in immunocompetent individuals. Until relatively recently leishmaniasis of any form was not known in South East Asia but it is now an emerging infectious disease in Thailand.\(^3\) To date, most of the reported cases have come from southern Thailand,\(^3\) and the first confirmed autochthonous case was reported from this region in 1999, presenting as visceral leishmaniasis in a 3-year-old girl native to Surat Thani Province and who had never been abroad.\(^4\) Since then, several further cases have been reported from southern Thailand, including some with HIV co-infection, and some of these with mixed symptomology of visceral and cutaneous leishmaniasis.\(^5-10\) The first case in northern Thailand, a 40-year-old man from Nan Province, was reported in 2005,\(^11\) and the second, a 52-year-old man from Lamphun Province, in 2012.\(^12\) Both of these cases presented with clinical manifestations of visceral leishmaniasis, and neither patient had any known underlying immunodeficiency or cutaneous presentation. Here we report two new cases from northern Thailand with different clinical features and cutaneous lesions.

**Materials and methods**

**Ethics**

Owing to ill health with undiagnosed conditions, the patients described herein were admitted to Maharaj Nakorn Chiang Mai hospital. All biopsy samples and other clinical investigations performed were part of routine clinical investigative procedures to determine the nature of their illnesses. No samples or procedures were undertaken for research purposes only. This report does not contain any identifiable information that could be used to compromise patient confidentiality.

**Parasite culture**

Promastigote cultures were initiated by inoculation of biopsy samples into 25-cm\(^2\) tissue culture flasks containing 5 mL Schneider’s *Drosophila* medium (Sigma-Aldrich, St Louis, MO, U.S.A.) supplemented with 20% (v/v) fetal bovine serum and maintained at 26 °C.\(^12\) Subsequently, they were also cultured in Medium 199 (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (Thermo-Fisher, Waltham, MA, U.S.A.) and BME vitamins (Sigma-Aldrich). Promastigotes were cryopreserved in 7.5% (v/v) glycerol in culture medium and stored at −80 °C and liquid nitrogen.

**Polymerase chain reaction and DNA sequencing**

DNA was isolated from parasites using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. Polymerase chain reaction (PCR) amplifications of various target sequences were performed as previously described: the ribosomal RNA internal transcriber spacer 1 (ITS-1) sequence with LeF/LeR primers;\(^13\) the large subunit of RNA polymerase II (RNApolII) gene with N7/N8 primers;\(^12\) and the heat shock protein 70 (HSP70) gene with HSP70sen/HSP70ant primers.\(^14\) The RPL23a intergenic sequence (IGS) was amplified with BN1 (GAA GGT CAA CAC CCT GAT CC) and BN2 (CTT CTT GGC GGT CTT CTG AG) primers. Amplification was performed with proofreading DNA polymerase (HotStar HiFidelity 124 Polymerase; Qiagen), 100 pmol of each primer and 100 ng parasite DNA. Initial denaturation was at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, annealing at various temperatures for 30 s (1 min for HSP70sen/HSP70ant), extension at 72 °C for 1 min (2 min for HSP70sen/HSP70ant) and a final extension at 72 °C for 10 min. Specific annealing temperatures were as follows: 57 °C for LeF/LeR; 57 °C for N7/N8; 61 °C for HSP70sen/HSP70ant; and 60 °C for BN1/BN2. Products were checked for size and purity by agarose gel electrophoresis and then directly sequenced or cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, U.S.A.) and sequenced using commercial services. Results were checked for quality using Chromas Lite 2.1.1 (http://technelysium.com.au/). Sequence alignments were performed using Clustal Omega (http://www.ebi.ac.uk/tools/msa/clustalo/).

**Results**

**Patient 1**

A 48-year-old man from Hang Dong District (Chiang Mai province, northern Thailand) had a 4-year history of skin nodules. He had worked as an industrial worker in southern Thailand in the early 1980s. He returned to his hometown 20 years ago and became a salesperson. During his career, he undertook short trips to various countries in South East Asia, including Indonesia, Cambodia and Singapore. In the early 2000s, he was diagnosed as having HIV. He ended his career and, owing to progression of the disease, received nevirapine, lamivudine and zidovudine for 6 years. Two years after antiretroviral treatment, he began to develop hyperpigmented nodules on his face and both elbows, and which later extended to both hips and legs. Upon admission to hospital in 2012, physical examination showed multiple discrete firm hypopigmented and brownish papules and nodules on his inner canthi, eyelids, nose, helices and antihelices of both pinnae, and extensor surfaces of hands, forearms and legs, particularly over the knuckles, elbows, ulnar ridges, knees, tubial crests and malleoli, buttocks, palms and soles (Figs 1–3). The patient’s liver and spleen were enlarged. Laboratory investigation revealed a haemoglobin level of 11.8 g dl\(^{-1}\), a white
blood cell (WBC) count of 3080 per mm\(^3\) and a platelet count of 97,000 per mm\(^3\). Liver function tests gave the following results: a serum albumin level of 2.9 g dL\(^{-1}\), a globulin level of 4.9 g dL\(^{-1}\), an aspartate aminotransferase (AST) level of 41 U L\(^{-1}\), an alanine aminotransferase (ALT) level of 20 U L\(^{-1}\) and an alkaline phosphatase (ALP) level of 100 U L\(^{-1}\). His CD4 T-cell count was 121 cells per mm\(^3\).

A skin biopsy was performed and histopathology showed normal epidermis and thickening of the dermal collagen (Fig. 4a). In the dermis, many yeast-like organisms were found between the collagen bundles and almost invariably separated from the epidermis by a narrow grenz zone of normal collagen (Fig. 4b); there was minimal inflammation. A fungal culture was negative but the patient was initially diagnosed with a deep fungal infection. He received oral itraconazole 400 mg daily for 1 year from a community hospital and showed slight improvement.

During follow-up investigations, skin smears and a biopsy continued to show yeast-like organisms, but Giemsa staining also showed the presence of a rod-shaped structure in their cytoplasm, near the nucleus, that was similar to a Leishmania kinetoplast, suggesting that the yeast-like organisms could be Leishmania amastigotes. The histopathological findings were similar to the previous biopsy. Many of the yeast-like organisms were also observed in a bone marrow examination. Cultures from skin and bone marrow specimens were prepared in Leishmania culture medium and Leishmania promastigote forms were observed after 3 days. This isolate was given the World Health Organization (WHO) designation MHOM/TH/2013/LSCM2. Following this parasitological diagnosis of leishmaniasis, the patient was treated with intravenous amphotericin B deoxycholate (1 mg kg\(^{-1}\) daily) for 20 days. Other therapeutic options such as sodium stibogluconate and miltefosine are not currently available in Thailand. Prophylactic intravenous fluid hydration was used; however, acute renal failure developed, which improved with continued intravenous fluid replacement. Subsequently, the patient has been receiving oral itraconazole 400 mg daily as maintenance therapy. Regression of the lesions was observed at 2 months after treatment (Figs 2 and 3).

In December 2014 the patient had 10 small, skin-coloured papules on the upper lip, the remnants of small lesions at the third metacarpophalangeal joints, and small, skin-coloured papules at the right elbow. He refused further investigation but was still on long-term oral itraconazole. PCR using the LeF/LeR primers still detected Leishmania DNA in the patient’s blood.

**Patient 2**

When working as a lumberjack, a 38-year-old man from Mae Tha district (Lumphun province, northern Thailand) started to develop brownish papules on the dorsum of both hands over a 4-year period. He noted that the lesions became enlarged and extended to both elbows and legs. Three years before admission he had burning sensations on the dorsum of both hands and changed career to become a wood craftsman. However, the lesions continued to progress to both palms and his face, and he also had intermittent fevers. The patient was diagnosed with HIV infection and has been receiving nevirapine, lamivudine and zidovudine since 2007. Upon admission to hospital in 2012 an immunological profile revealed a CD4 T-cell count of 543 cells per mm\(^3\). The viral load was undetectable (< 20 copies mL\(^{-1}\)). He had never travelled abroad.

Physical examination showed multiple discrete hypopigmented papules and nodules at the inner and outer canthi of eyes, helices and antihelices of both pinnae, and extensor surfaces of hands, forearms and legs, particularly over the knuckles, elbows, ulnar ridges, knees, tibial crests and malleoli, and multiple hypopigmented sclerotic plaques on his palms. His liver and spleen were not enlarged. Blood analysis revealed a haemoglobin level of 10·6 g dL\(^{-1}\), a WBC count of 7200 per mm\(^3\) and a platelet count of 100,000 per mm\(^3\). The results of his liver function tests showed a serum albumin level of 3·0 g dL\(^{-1}\), a globulin level of 6·0 g dL\(^{-1}\), an AST level of 67 U L\(^{-1}\), an ALT level of 54 U L\(^{-1}\) and an ALP level of 329 U L\(^{-1}\). Abdominal ultrasonography revealed increased echogenicity of liver and renal parenchyma.

The smears and histopathology of skin lesions and bone marrow revealed similar organisms to patient 1 and therefore a diagnosis of leishmaniasis was suspected. Skin and bone marrow samples were inoculated into culture and, after a few days of incubation, were positive for Leishmania promastigotes. This isolate was given the WHO designation MHOM/TH/2013/LSCM3. Intravenous amphotericin B deoxycholate (1 mg kg\(^{-1}\) daily) was prescribed with prophylactic intravenous fluid hydration. Within 3 days of treatment, the patient developed acute renal failure, which improved with continued intravenous fluid hydration. Oral itraconazole at 200 mg daily was started after completion of a 2-week course of amphotericin B for ongoing suppressive therapy. The skin lesions improved from 1 week after the treatment and a relapse has not been observed.

**Molecular characterization**

Cultures of Leishmania promastigotes were established using biopsy samples from patients 1 and 2, and named as isolates LSCM2 and LSCM3, respectively. DNA was extracted from each
isolate and analysed by PCR amplification and sequencing, comparing the data obtained with those obtained using a previously characterized isolate of *Leishmania martiniquensis* from Thailand that caused visceral leishmaniasis (LSCM1)\(^1\) and the reference strain of *L. martiniquensis*, LEM2494 (MAR1), originally from Martinique.\(^1\) Sequences from four independent loci were examined: the ITS-1 region of the 18S ribosomal RNA gene (sequence located on reference genome *Leishmania major* chromosome 27); the large subunit of RNAPolII (*L. major* chromosome 31); the HSP70 gene (*L. major* chromosome 28); and the IGS between two tandemly repeated RPL23a genes (*L. major* chromosome 6). These loci have all been previously used for *Leishmania* identification.\(^1\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\) The accession numbers for the newly generated sequences and reference sequences from Genbank are given in Table 1, and sequence alignments are given in Appendix S1 (see Supporting Information). In each case, the sequences obtained for LSCM2 and LSCM3 were identical to each other: 259 base pairs (bp) for ITS-1; 532 bp for RNAPolII; 1316 bp for HSP70; and 472 bp for RPL23a IGS. Further, these sequences were also identical

Fig 2. Skin lesions on the dorsum of both hands. (a) Immediately before treatment showing presence of lesions particularly on the knuckles. (b) Two months later after treatment with amphotericin B and itraconazole.

Fig 3. Skin lesions on the elbows and extensor surfaces of the forearms. (a) Immediately before treatment showing lesions particularly over the ulnar ridge. (b) Two months later after treatment with amphotericin B and itraconazole.

Fig 4. Histopathology of skin biopsy taken from the right forearm. (a) Low magnification photograph demonstrating the overall normal appearance of the epidermis but with thickening of the dermal collagen. Many yeast-like organisms were found in the dermis and were almost invariably separated from the epidermis by a narrow grenz zone of normal collagen (haematoxylin and eosin, 40 ×). (b) Higher magnification picture of the inset of (a) showing many yeast-like organisms infiltrating between the collagen bundles but with minimal inflammation (haematoxylin and eosin, 400 ×).
to those from LSCM1 and LEM2494. Therefore, we concluded that both patients were infected with L. martiniquensis.

Discussion

Here we report two HIV-positive patients who presented with the clinical features of disseminated cutaneous and visceral leishmaniasis. Cultures were established and the parasite was identified by DNA sequencing of four independent loci as the recently described species Leishmania martiniquensis. L. martiniquensis is named after the Caribbean island of Martinique, where it was originally discovered and where it mainly causes cutaneous leishmaniasis. The current report constitutes the first confirmed cases of cutaneous leishmaniasis due to L. martiniquensis in Thailand and outside Martinique.

Since the onset of the AIDS epidemic there has been much concern regarding opportunistic infections in HIV-infected immunocompromised individuals, and leishmaniasis is one of several diseases that are more severe in such individuals. Indeed, there is evidence that in addition to being an opportunistic infection, visceral leishmaniasis also predisposes individuals to HIV infection, creating a highly undesirable co-infection scenario. Thus, to date, co-infection of leishmaniasis and HIV has been mostly reported in cases of infection with Leishmania infantum, the major causative agent of visceral leishmaniasis in Europe and South America, and Leishmania donovani, the agent of visceral leishmaniasis in Africa and the Indian subcontinent. Leishmania martiniquensis is a recently described species, formally named in 2014, which is found in a range of clinical presentations, and can also present in some patients as visceral leishmaniasis. Leishmania martiniquensis is a taxonomically invalid name (nomen nudum); however, as this study and that of Pothirat et al. show, these cases of 'L. siamensis' infection are probably due to L. martiniquensis. Confirmation of this requires further analysis by the researchers concerned. As L. martiniquensis has only recently been recognized and formally described, it probably has a wider geographical spread than is apparent from the available case reports – the predominance reported from Martinique and Thailand probably reflecting a heightened awareness of the infection in these countries. So far it has caused disease relatively rarely in humans, although as an emerging infection this may change, and dermatologists need to be aware of the clinical presentations and potentially wide geographical distribution of the infection. The high relative frequency of detection in HIV-infected individuals suggests the infection is usually nonpathogenic in the majority of immunologically intact individuals, and has an established life cycle with possibly novel vectors and reservoir hosts that remain to be determined.

Cutaneous involvement has been reported to occur in approximately 2–12% of patients co-infected with L. infantum/ L. donovani and HIV. Previously reported cases of leishmania-HIV co-infection in Thailand revealed manifestations of disseminated visceral and cutaneous leishmaniasis, which were similar to those seen in our patients. In addition to the disseminated presentation described herein, localized cutaneous leishmaniasis due to L. martiniquensis has also been seen in immunocompetent patients, involving solitary papulo-nodule lesions with or without ulceration on the face localized on the periorbital area, forehead and ear lobe, which spontaneously healed after several months. Cutaneous manifestation of leishmaniasis in patients co-infected with HIV is associated with clinical polymorphism and a challenging differential diagnosis, especially in nonendemic areas. Various manifestations have been reported, including papulonodular, ulcerative, infiltrative, lepromatous and diffuse, psoriasis-like, keloid, histoid, Kaposi sarcoma-like or dermatofibroma-like lesions. Such lesions were mostly observed on the face, extremities and acral area. Our patients showed similar skin lesions to each other that included chronic generalized fibrotic papules and nodules distributed on the face, ear, trunk and extremities. The lesions appeared to be more prominent on the traumatic areas, for example on skin over the joints, bony prominences, ear cartilage, palms and soles, and in a linear distribution on the skin over the ulnar ridges.

Table 1 Accession numbers of sequences used for comparison of isolates

| Isolate            | Sequence          | RNAPolII | HSP70   | RPL23a  |
|--------------------|-------------------|----------|---------|---------|
| Leishmania martiniquensis LEM2494 | KM677931 KM820663 | KP244365  | KP025945 |
| Leishmania martiniquensis LSCM1    | JX899838 KM677933 | KP244366  | KP244362  |
| LSCM2               | KJ210834^ KM210835a | KP244367^ | KP244363^  |
| LSCM3               | KJ210836^ KJ210837a | KP244368^ | KP244364^  |

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The diversity of clinical manifestations of cutaneous leishmaniasis depends not only on the infecting strains, but also on the nature of the host immune response. In immunocompetent hosts, a Th1 helper cell (Th1) response is associated with a protective effect against Leishmania, while susceptibility to infection and disease progression are associated with Th2 cytokines.19 HIV infection promotes a Th2 immune response that facilitates the progression of leishmaniasis.30 Decreased interferon-γ, interleukin (IL)-12 and IL-18 production was also induced fibroblasts to produce collagen, which may be responsible for the fibrotic lesions on the face, ear, trunk and extremities that were more prominent on the traumatic areas. Observation of fibrotic lesions on the face, ear, trunk and extremities that were more prominent on the traumatic areas. Observation of fibrotic lesions seen in our patients.32

Treatment of leishmaniasis in patients infected with HIV is complicated by lower cure rates, higher drug toxicity and higher fatality rates than in patients not infected with HIV. However, there is a higher clinical improvement rate when using amphotericin B than pentavalent antimonial compounds.33 Initial cure rates with amphotericin B ranged from 58% to 82%, with frequent relapses; however, renal toxicity occurred in 18–36% of patients. Our patients were treated with intravenous amphotericin B and showed a significant reduction in lesion number. Acute renal failure was a complication, which improved with intravenous fluid hydration in both patients. Itraconazole is an antifungal agent that inhibits sterol synthesis in Leishmania and disrupts the growth and division of amastigotes.34 Some case reports and studies have indicated that itraconazole can be an effective treatment for cutaneous leishmaniasis.24,35,36 Nevertheless, a large randomized study did not demonstrate the efficacy of itraconazole over placebo,37 and it is not currently considered a first-line drug for the treatment of leishmaniasis. Before the parasitological diagnosis, patient 1 was treated with oral itraconazole for a year, with slight improvement, for suspected fungal infection. However, owing to the low response rate, itraconazole should not be used as a single agent in the treatment of cutaneous leishmaniasis.38 Because of a high relapse rate in patients co-infected with HIV, maintenance therapy was considered to prevent early relapse. Itraconazole was shown to be effective as a suppressive therapy in our patients and in some other case reports.39,40

In conclusion, this is the first confirmed report of autochthonous disseminated cutaneous and visceral leishmaniasis in Thailand caused by L. martiniquensis. The manifestations of the cutaneous lesions were characterized by chronic generalized fibrotic lesions on the face, ear, trunk and extremities that were more prominent on the traumatic areas. Observation of yeast-like organisms in skin biopsies should increase the awareness of a possible diagnosis of leishmaniasis, even in nonendemic areas. Owing to the lack of availability of pentavalent antimonial compounds, amphotericin B is the main therapeutic option in Thailand, but is an effective treatment for leishmaniasis. Leishmania martiniquensis appears to be an aetiological agent of cutaneous leishmaniasis in Thailand.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

 Appendix S1. Alignment of ITS-1, RNAPolII, HSP70 and RPL23a sequences.