Case Report: Confirmation by Metagenomic Sequencing of Visceral Leishmaniasis in an Immunosuppressed Returned Traveler

Eloise Williams,^{1,2*} Nicole S. Isles,³ Torsten Seemann,³ Trevor Kilpatrick,^{4,5,6} Andrew Grigg,⁷ Marcel Leroi,^{1,2} Benjamin P. Howden,^{1,3} and Jason C. Kwong^{1,3}

¹Department of Infectious Diseases, Austin Health, Heidelberg, Australia; ²Department of Microbiology, Austin Health, Heidelberg, Australia; ³Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology and Immunology, The University of Melbourne, Peter Doherty Institute for Infection and Immunity, Parkville, Australia; ⁴The Florey Institute of Neuroscience and Mental Health, Parkville, Australia; ⁵Department of Anatomy and Neuroscience, The University of Melbourne, Parkville, Australia; ⁶Department of Neurology, Royal Melbourne Hospital, Parkville, Australia; ⁷Department of Clinical Haematology, Austin Health, Heidelberg, Australia

Abstract. There has been increased interest in using metagenomic next-generation sequencing as an unbiased approach for diagnosing infectious diseases. We describe a 61-year-old man on fingolimod therapy for multiple sclerosis with an extensive travel history who presented with 7 months of fevers, night sweats, and weight loss. Peripheral blood tests showed pancytopenia and abnormal acute phase reactants. A bone marrow aspirate showed the presence of numerous intracellular and extracellular amastigotes consistent with visceral leishmaniasis (VL). Metagenomic sequencing of the bone marrow aspirate confirmed *Leishmania infantum*, a species widely reported in the Mediterranean region. This correlated with acquisition of VL infection during the patient's most recent epidemiological exposure in southern Italy 12 months prior. This case demonstrates the potential application of metagenomic sequencing for identification and speciation of *Leishmania* in cases of VL; however, further assessment is required using other more readily obtained clinical samples such as blood.

CASE REPORT

A 61-year-old Australian man presented with 7 months of fevers, night sweats, fatigue, and weight loss. His most recent travel was 12 months prior, comprising a 3-week trip to southern Italy; he had traveled extensively throughout Europe and Asia in the past 10 years, including a trip to India 6 years prior. His medical history was notable for relapsing remitting multiple sclerosis (MS) managed with fingolimod for the past 6 years and recent ex-smoking.

He looked unwell and was febrile at 39.2°C; examination showed mild non-tender hepatomegaly without palpable splenomegaly or other abnormal findings. Laboratory workup revealed pancytopenia with a hemoglobin level of 121 g/L (reference range [RR]: 130–170 g/L), platelets of 130×10^9 /L (RR: $150\text{--}400 \times 10^9$ /L), and a white cell count of 2.6×10^9 /L (RR: $4\text{--}12 \times 10^9$ /L) with a lymphocyte count of 0.3×10^9 /L (RR: $1.5\text{--}3.5 \times 10^9$ /L) and a neutrophil count of 1.7×10^9 /L (RR: $2.0\text{--}8.0 \times 10^9$ /L). Blood film was nondiagnostic. Acute phase reactants were also abnormal, with an erythrocyte sedimentation rate of 45 mm/hour, C-reactive protein of 26 mg/L (RR: 0–5), albumin of 30 g/L (RR: 35–50 g/L), ferritin of 3,072 µg/L (RR: 30–400 µg/L), and polyclonal hypergammaglobulinemia. An abdominal ultrasound showed mild hepatomegaly. HIV antigen–antibody complex was nonreactive. Lymphocyte subsets were reduced, with a CD4⁺ lymphopenia of 0.23×10^9 /L (RR: $0.65\text{--}2.00 \times 10^9$ /L) and CD8⁺ lymphopenia of 0.21×10^9 /L (RR: $0.33\text{--}1.31 \times 10^9$ /L). Retrospective review since commencement of fingolimod therapy 6 years prior showed a persistent lymphopenia with a total lymphocyte count between 0.2 and 0.3×10^9 /L.

Given the pancytopenia, a bone marrow aspirate and trephine were performed with the expectation of finding an aggressive hematological malignancy. Unexpectedly, the

aspirate revealed numerous intracellular and extracellular *Leishmania* amastigotes (Figure 1A and B). Whole community shotgun metagenomic sequencing of DNA extracted from the aspirate was undertaken on the Illumina NextSeq, generating 121,364,564 sequencing reads. After removing human genome sequences, 905,743/121,364,564 (0.7%) nonhuman sequencing reads remained, of which 294,673/905,743 (32.5%) were classified as *Leishmania* reads. No *Leishmania* reads were identified in the negative control sample sequenced concurrently. PCR targets for small subunit ribosomal ribonucleic acid (rRNA), kinetoplast minicircle DNA, and the conserved *Leishmania infantum* DNA repeat region (REPL-repeat) region of the *Leishmania* genome were identified in silico from the metagenomic sequencing data (see Supplemental Appendix). The species, *Leishmania infantum*, was confirmed by in silico reconstruction of the *hsp70* gene and whole-genome phylogenetic comparisons of the reconstructed draft genome sequences with publicly available *Leishmania* genomes (Figure 1C). This correlated with acquisition of infection during the patient's most recent epidemiological exposure in southern Italy 12 months prior, rather than the possible exposure to *Leishmania donovani* in India 6 years prior.

After the diagnosis of visceral leishmaniasis (VL) was confirmed and on the basis that profound CD4 lymphopenia was a likely contributing factor, fingolimod was ceased. Treatment consisted of 40 mg/kg liposomal amphotericin B, which was given over 2 months. His symptoms, pancytopenia, and inflammatory markers rapidly resolved, and he remains well 6 months after treatment cessation. The CD4 lymphopenia resolved with a count of 0.73×10^9 /L 3 months after cessation of fingolimod. His MS has remained stable, except for the subsequent development of trigeminal neuralgia without evidence of new central nervous system lesions, including at the root entry zone of the trigeminal nerve in the pons. He remains off immunomodulatory therapy.

DISCUSSION

Visceral leishmaniasis is a vector-borne parasitic disease caused by obligate intracellular protozoa of the genus

* Address correspondence to Eloise Williams, Department of Microbiology and Infectious Diseases, Austin Health, 145 Studley Rd., Heidelberg 3084, Australia. E-mail: eloise.williams@austin.org.au

FIGURE 1. (A) Romanowsky stain of bone marrow aspirate, original magnification $\times 1,000$: Leishman-Donovan bodies; numerous intracellular and extracellular *Leishmania* amastigotes are seen. (B) Romanowsky stain of bone marrow aspirate, original magnification $\times 1,000$: *Leishmania* amastigotes are oval, with a round nucleus (red arrow) and rod-like kinetoplast (black arrow). (C) Maximum likelihood phylogenetic comparison of the reconstructed *Leishmania* genome from the patient sample (red arrow) with publicly available *Leishmania* genomes. The reconstructed genome was most closely grouped with *Leishmania infantum*. Numbers show branch support from 1,000 bootstrap replicates.

Leishmania.¹ Visceral leishmaniasis is transmitted between mammalian hosts by 1.5–2 mm female phlebotomine sand flies, and is typically caused by *L. donovani* in Asia and Eastern Africa and *L. infantum* in Latin America, Central Asia, and the Mediterranean. The incubation period is between 2 weeks and 8 months; however, symptoms may occur years later in infected persons after they become immunosuppressed.¹

In immunocompetent hosts, *Leishmania*-specific immune response is associated with a T-helper 1 cytokine response, including interleukin (IL)-12, interferon (IFN) gamma, and tumor necrosis factor alpha (TNF- α) secretion, thereby recruiting and activating macrophages that phagocytose cells infected with leishmanial amastigotes.² It is not surprising, therefore, that T-cell immune deficiency is known to be a risk factor for VL, with patients with HIV having 100–2,320 times the risk of developing VL compared with immunocompetent hosts.³ Solid organ transplantation, hematological and solid organ malignancies, and TNF- α antagonist therapy have also been associated with an increased risk of VL.⁴ In addition to increasing the risk of VL, immunosuppression is also associated with reduced response rates to VL treatment and an increased risk of relapse.⁴ Treatment recommendations therefore differ for immunocompromised persons, with a higher total dose (40 mg/kg versus 21 mg/kg) and a longer

treatment duration of liposomal amphotericin B recommended in these patients.⁴

To our knowledge, this is only the second reported case of VL in the context of fingolimod therapy for MS.⁵ Fingolimod is a sphingosine-1-phosphate (S1P) receptor inhibitor which entraps lymphocytes within lymph nodes, reducing infiltration of aggressive lymphocytes into the central nervous system.⁶ It was approved for the management of MS in 2009 and is often used as first-line therapy. It preferentially entraps C-C receptor 7 (CCR7)-expressing naive and central memory lymphocytes within lymph nodes, without significantly effecting circulation of CCR7—effector memory T cells.⁷ Fingolimod treatment results in persistent peripheral blood lymphopenia involving both CD4⁺ and CD8⁺ cells, with a total lymphocyte count reduction of 73% compared with baseline noted after the first month of therapy in phase 3 trials.⁶

Despite its lymphopenic effects, fingolimod has not been reported to significantly increase infections compared with control populations in phase 3 trials, attributed to the apparent absence of the effect of fingolimod on effector memory T cells. However, review of clinical trial and post-marketing surveillance data has demonstrated an increased risk of varicella zoster virus (VZV) infection,⁸ John Cunningham virus causing progressive multifocal leukoencephalopathy,

and cryptococcal infection⁹ in fingolimod-treated patients. Further studies have also demonstrated reduced production of pro-inflammatory cytokines IFN- γ and IL-17 by CD4⁺ and CD8⁺ cells¹⁰ in the presence of fingolimod. Sphingosine-1-phosphate signaling also appears to be significant in the host response to *Leishmania* infection, with inhibition of S1P associated with increased IL-10 production and increased intracellular parasite burden.¹¹ Varicella serology is required before commencement of fingolimod, and VZV vaccination should be given at least 1 month before therapy in seronegative patients. There are currently no recommendations regarding prophylaxis or monitoring for other opportunistic infectious diseases for patients on fingolimod; however, clinicians should be aware of these associations and investigate specifically for opportunistic infections in patients who present with unusual symptoms while on fingolimod.

Diagnosis of VL can be difficult in those presenting in non-endemic areas such as returned travelers, particularly in immunosuppressed patients who often present atypically. A recent review of 28 imported cases of VL in the United Kingdom over an 18-year period found that imported cases were more likely to occur in those who were immunosuppressed (68%), with a rise of VL in immunocompromised patients because of immunomodulatory drugs for autoimmune diseases compared with previous data. Tourist travel to a VL-endemic region (57%) and travel to the Mediterranean basin (75%) were also risk factors.² The median time from first symptom to diagnosis was 5 months in the immunosuppressed group, with a range of 1–120 months, demonstrating the diagnostic difficulty of VL in these patients.

A number of diagnostic methods for VL are available and have been reviewed in detail elsewhere, with each method having variable performance characteristics with regard to sensitivity, specificity, speed, accessibility, ease of use, cost, and level of discrimination of *Leishmania* species.¹² In the context of this patient, for whom the differential diagnosis at presentation included both infectious and noninfectious diseases, microscopic examination of the bone marrow aspirate allowed for an early and accurate preliminary diagnosis, supported by metagenomic sequencing to confirm the diagnosis and *Leishmania* species.

The rationale for using metagenomic sequencing to confirm the diagnosis and *Leishmania* species was 2-fold. First, the *Leishmania* PCR available in our diagnostic laboratory uses an internal transcribed space region 1 (ITS1) target that is unable to discriminate between *L. donovani* and *L. infantum* species. PCR assays based on *hsp70* have the ability discriminate between these species; however, these are not available in diagnostic laboratories in Australia. Our second objective was to evaluate the application of metagenomic sequencing in the diagnosis of VL. To our knowledge, this is the first reported case where metagenomic sequencing has been used for the detection of *Leishmania* infection. There has been increased interest in using metagenomic sequencing as a hypothesis-free, unbiased approach for diagnosing infectious diseases, with the potential for this approach to lead to a significant paradigm shift from the current diagnostic practice of performing numerous individual tests directed at specifically targeted organisms.¹³ Although in this case *L. infantum* was detected on a bone marrow aspirate where the organisms were concentrated and apparent on microscopic examination, this proof-of-principle example suggests that metagenomic sequencing has the potential to identify *Leishmania*

species in other more accessible clinical samples such as blood. In the context of this patient from a non-endemic area who had a number of epidemiological exposures and long period of immunosuppression, we were able to infer from the sequencing data that he most likely acquired VL during his travel to the Mediterranean basin. This has implications for the choice of potentially required second-line therapy, with pentavalent antimonials no longer recommended in *L. donovani* infection acquired in India because of drug resistance.¹

Although the current costs of metagenomic sequencing are high and its sensitivity compared with PCR is so far untested, this case demonstrates that it can successfully detect *Leishmania* infection. It is unlikely that metagenomic sequencing will replace microscopy or PCR for the diagnosis of *Leishmania* in endemic countries; however, it has the potential to be used in complex cases, particularly in immunocompromised patients with systemic illness where the differential diagnosis is broad or multiple pathogens may be implicated in disease.

This case of fingolimod-associated VL in a returned traveler from Italy illustrates the need for vigilance for the emergence of rare, opportunistic infections in patients on novel immunosuppressive agents, particularly following travel to endemic areas. The application of metagenomic sequencing has the potential to identify and speciate *Leishmania* in cases of VL; however, further assessment is required using more readily obtained clinical samples such as blood.

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Authors' addresses: Eloise Williams and Marcel Leroi, Department of Infectious Diseases, Austin Health, Heidelberg, Australia, and Department of Microbiology, Austin Health, Heidelberg, Australia, E-mails: eloise.williams@austin.org.au and marcel.leroi@austin.org.au. Nicole S. Isles and Torsten Seemann, Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology and Immunology, The University of Melbourne, Peter Doherty Institute for Infection and Immunity, Parkville, Australia, E-mails: isles.n@unimelb.edu.au and t.seemann@unimelb.edu.au. Trevor Kilpatrick, The Florey Institute of Neuroscience and Mental Health, Parkville, Australia, Department of Anatomy and Neuroscience, The University of Melbourne, Parkville, Australia, and Department of Neurology, Royal Melbourne Hospital, Parkville, Australia, E-mail: tkilpat@florey.edu.au. Andrew Grigg, Department of Clinical Haematology, Austin Health, Heidelberg, Australia, E-mail: andrew.grigg@austin.org.au. Benjamin P. Howden and Jason C. Kwong, Department of Infectious Diseases, Austin Health, Heidelberg, Australia, and Microbiological Diagnostic Unit Public Health Laboratory, Department of Microbiology and Immunology, The University of Melbourne, Peter Doherty Institute for Infection and Immunity, Parkville,

Australia, E-mails: bhowden@unimelb.edu.au and jason.kwong@austin.org.au.

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