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Succinctus

First isolation of a new species of *Leishmania* responsible for human cutaneous leishmaniasis in Ghana and classification in the *Leishmania enriettii* complex

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ABSTRACT

An active case detection approach with PCR diagnosis was used in the Ho District of the Volta Region, Ghana that identified individuals with active cutaneous leishmaniasis. Three isolates were successfully cultured and DNA sequences from these were analysed (ribosomal RNA internal transcribed spacer 1; ribosomal protein L23a intergenic spacer; RNA polymerase II large subunit), showing them to be *Leishmania*, identical to each other but different from all other known *Leishmania* spp. Phylogenetic analysis showed the parasites to be new members of the *Leishmania enriettii* complex, which is emerging as a possible new subgenus of *Leishmania* parasites containing human pathogens.

Human cutaneous leishmaniasis (CL) is a significant emerging disease in the Volta Region of Ghana (Kweku et al., 2011) and has become sufficiently common to acquire a local name, "agbamekanu", with estimates of high prevalence in some communities. Reported infections have occurred mainly in the Ho District, a moist semi-deciduous forest zone with villages dotted around the district capital and an estimated population of 271,881 (Ghana Statistical Service, 2010 Population and Housing Census http://www.statsghana.gov.gh/docfiles/2010phc/2010_POPULATION_AND_HOUSING_CENSUS_FINAL_RESULTS.pdf). From clinical signs, 8,533 cases were recorded in the Ho District in 2002 and 2003, which represents approximately 3–4% of the population, and in surveys of schoolchildren prevalence of leishmanial-like lesions ranged from 12.2% to 32.3% Kweku et al., 2011). However, the number of cases with parasitologically confirmed diagnoses is very small (Fryauff et al., 2006; Villinski et al., 2007), and prior to the current study parasites causing CL in Ghana had never been isolated into culture. The identity of the species responsible remains uncertain. One PCR-confirmed case from a biopsy was identified as *Leishmania major* by rRNA internal transcribed spacer 1 (ITS1) sequencing (Fryauff et al., 2006), but in a second study conducted in the same area further biopsies found no match to any known *Leishmania* sp. (Villinski et al., 2007). Regarding transmission, leishmaniasis is a vector-borne disease usually transmitted by sand flies but the majority caught to date in Ghana have been various *Sergentomyia* spp., which are not generally regarded as likely vectors of human leishmaniasis (Ready, 2013). However, low numbers of the possible vectors, *Phlebotomus rodhaini* and *Phlebotomus duboscqi*, were found (Fryauff et al., 2006). It has been recently reported that *Leishmania tropica* DNA was found in *Sergentomyia*

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hamoni and *Sergentomyia ingrami*, and *L. major* DNA in *S. ingrami*, in flies collected from the endemic region (Nzelu et al., 2014). However, the finding of parasite DNA by itself does not prove vector status, as blood meal infections can persist in non-vectors for some days but will not result in established transmissible infections (Ready, 2013). The current study was undertaken to isolate and characterise parasites causing CL in Ghana. Cultures were established for the first time, and here we present evidence that these represent a new species of *Leishmania*, which is related to several other species grouped within the *Leishmania enriettii* complex. To our knowledge, these parasites are the first new human-infective *Leishmania* spp. to be isolated in Africa for over 40 years.

Fifteen villages in the Ho District with previous records of suspected or reported cases of CL were initially visited, and of these five villages with recent cases were followed up: Matse-Lotus, Sokode-Gbogame, Dodome-Doglome, Dodome-Awiausu and Lume-Atsyame. The study was assessed by, and approved by, the University of Ghana Medical School Ethical and Protocol Review Board, Protocol Identification Number MS-Et/M.6.1–P.3/2006-07 and the Noguchi Memorial Institute for Medical Research Institutional Review Board, Ghana, CPN 062/11-12. The purpose of the study and the procedures to be followed were explained and written informed consent was obtained from all participants or their legal guardians prior to any intervention. A total of 68 people with suspected CL were seen, of these 44 were recruited into the study and 41 subsequently confirmed as infected with *Leishmania*, 38 by PCR from dermal scrapings and three cases by successful culture and DNA sequence analysis (see Supplementary Data S1 for further details of sampling and other methods). Typical households included 10–14 inhabitants, and usually 1–2 people per household had healed lesions on the body suggestive of past CL. Two sets of PCR primers were used for diagnosis: Mincr2 and Mincr3 are derived from the conserved region of *Leishmania* spp. minicircle DNA of the parasite kinetoplast, generating a product of 120 bp (Degrave et al., 1994; da Silva et al., 2004); primers R221 and R332 are *Leishmania*-specific and amplify a region of the 18S rRNA gene, generating a product of 603 bp (van Eys et al., 1992; Meredith et al., 1993). The Mincr2/Mincr3 primer pair demonstrated better efficacy, amplifying 38 out of the 41 dermal scraping samples taken (93%; Fig. 1A, Supplementary Fig. S1A), with the R221/R332 pair amplifying 27 out of the 41 samples (66%; Fig. 1B, Supplementary Fig. S1B). All samples that were positive with R221/R332 were also positive with Mincr2/Mincr3; no additional positive cases were discovered with R221/R332. Thus the R221/R332 primers detected *Leishmania* in fewer samples, which is consistent with the lower copy number of their targets at about 160 copies per genome (van Eys et al., 1992), compared with ~10,000 copy number of the minicr1/minicr2 targets (Degrave et al., 1994). The three negative samples from dermal scrapings are either true negative non-leishmanial skin lesions that can arise from a number of other causes, or are *Leishmania* infections below the level of detection. There was no evidence of other differential diagnoses from Buruli ulcer, yaws or cutaneous fungal infections amongst the participants. Use of dermal scrapings for diagnosis of *Leishmania* is a minimally invasive sampling method for CL, and another useful aspect of this study was the ability of the primers to detect *Leishmania* from the lesion material on FTA® cards (Whatman Bioscience Ltd), without the need for separate isolation of DNA from clinical samples.

Three lesion aspirate samples from separate individuals were used to establish promastigote cultures. Sloppy Evans semi-solid medium was prepared by mixing 350 ml of Locke's solution (9 g of NaCl, 0.42 g of KCl, 0.4 g of CaCl, 0.2 g of $NaHCO_3$, 1 g of glucose and dH_20 to 1 L) with 1.3 g of Agar No 1, 2 g of bacteriological peptone, and 0.2 g of beef extract (Bovril, UK), and autoclaved.

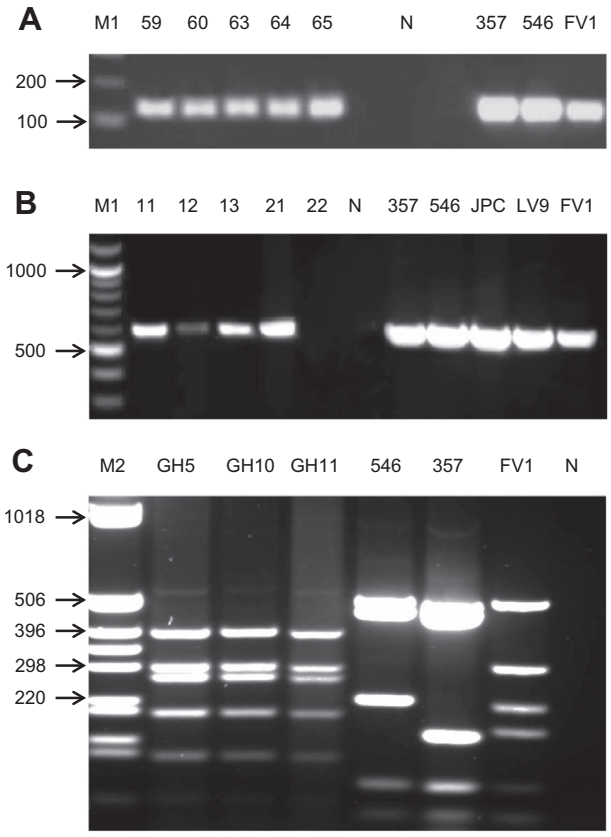

Fig. 1. PCR diagnosis and restriction fragment length polymorphism analysis of DNA extracted from human isolates of *Leishmania*. (A) An example of diagnosis using kinetoplast (k)DNA minicircle primers. Lesions were cleaned with 70% alcohol and scrapings stored on FTA® cards (Whatman BioScience, UK). Three 2 mm discs from each FTA® sample were processed for PCR and the products examined by agarose gel electrophoresis. Numbers 59–65 refer to participant sample numbers; M1 is a 100 bp ladder marker; N is a negative control; 357, 546 and FV1 are positive controls for *Leishmania tropica*, *Leishmania aethiopica* and *Leishmania major*, respectively. (B) An example of diagnosis using 18S rRNA primers. Numbers 11–22 refer to participant sample numbers; M1 is a 100 bp ladder marker; N is a negative control; 357, 546, JPC, LV9 and FV1 are positive controls for *L. tropica*, *L. aethiopica*, *Leishmania infantum*, *Leishmania donovani* and *L. major*, respectively. (C) Analysis of isolates by PCR-restriction fragment length polymorphism. DNA was purified from cultures of isolates GH5, GH10, GH11 and positive controls, amplified using primers AM1/AM2, and products digested with restriction enzyme *Msp*I. M2 contains size markers as indicated in bp; 546, 357 and FV1 are controls for *L. aethiopica*, *L. tropica* and *L. major*, respectively; N is a negative control. Further technical details are given in Supplementary Data S1, and further results in Supplementary Fig. S1.

Defibrinated sterile rabbit blood (50 ml) was added, mixed and 2 ml aliquots were dispensed into sterile Bijou tubes. Lesion aspirates were transferred into 2 ml volumes of Sloppy Evans medium, incubated at 26 °C and checked every 48 h for up to 1 month by phase contrast microscopy. Upon observing promastigotes, aliquots were transferred into liquid culture medium comprised of Medium 199 (12350-039, Life Technologies, UK) supplemented with 20% (v/v) FCS, BME vitamins (B6891, Sigma–Aldrich, UK) and 25 μg/ml of gentamicin sulphate. Cultures were expanded and sub-passaged as required and promastigotes cryopreserved in 7.5% glycerol at −80 °C and liquid nitrogen. The World Health organisation (WHO) codes for these isolates are MHOM/GH/2012/GH5;LV757, MHOM/GH/2012/GH10;LV758 and MHOM/GH/2012/GH11;LV759, hereafter referred to as GH5, GH10 and GH11, respectively. Amplification of the ITS1 sequence from each isolate was performed, and the resulting sequences were found to be very similar or identical to each other and to the sequence previously