

Short Report: Clinical and Molecular Evidence for a Case of Buruli Ulcer (*Mycobacterium ulcerans* Infection) in Kenya

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Abstract. *Mycobacterium ulcerans* infection is an emerging disease that causes indolent, necrotizing skin lesions known as Buruli ulcer (BU) and occasional contiguous or metastatic bone lesions. Buruli ulcer is named after Buruli County in Uganda (east Africa), where an epidemic occurred in the 1960s. Today, BU is most common in central and west Africa. We describe clinical and molecular evidence for a case of BU in Kenya.

Mycobacterium ulcerans is an emerging infection that causes indolent, necrotizing skin lesions known as Buruli ulcer (BU).¹ Reactive osteitis or osteomyelitis beneath skin lesions, or metastatic osteomyelitis from lymphohematogenous spread of *M. ulcerans*, develops in approximately 10% of infected patients.² The most plausible mode of transmission is by trauma at cutaneous sites superficially contaminated by *M. ulcerans*. Mycolactones elaborated by *M. ulcerans* are probably the most important pathogenic factors in lesions of BU.^{3,4} Incidence of BU is highest in children ≤ 15 years of age, and is a public health problem in disease-endemic countries because of disabling sequelae that may include scarring contractures, bone destruction, and amputations.² The prevalence of BU is highest in west and central Africa, but the disease has also been reported in more than 30 countries, including several countries in east Africa, such as Sudan.² We describe clinical and molecular evidence in support of the first confirmed case of BU in Kenya.

A 34-year-old woman (FO) who lived in a village approximately 10–15 km from Kisumu (Lake Victoria region in western Kenya) came to the outpatient clinic of New Nyanza Provincial General Hospital with four annular, painless, pristine ulcers with undermined borders and cotton-like necrotic centers on the right thigh (Figure 1). Each ulcer was surrounded by induration, mild hyperpigmentation, and scaling. The lesions had been present for 6–12 months and described as non-healing and slowly progressive. The hip area was tender on palpation and the patient walked with a limp. She did not recall antecedent trauma to the site. There was no evidence of previous treatment. Social history was notable for manual work in the family garden, and exposure to natural water sources when washing clothes. Serologic status for infection with human immunodeficiency virus was unknown.

Gram and Ziehl-Neelsen stainings of swab smears of exudate from the undermined areas of the ulcers showed gram-positive cocci and numerous, scattered, acid-fast bacilli (AFB) (Figure 2). Swab samples of exudate were placed in 70% ethanol for molecular analysis. Bacterial cultures or a lesional biopsy were not done. The patient was referred for a right hip radiograph to establish possible bone involvement, but she absconded and the procedure was not done. Upon completion

of molecular analyses described below, repeated attempts to find the patient for follow up were unsuccessful.

For real-time and conventional diagnostic polymerase chain reaction (PCR), DNA from lesion swabs of two patients, the subject of this report (FO), another local person (HA) who had a nonspecific AFB smear-negative ulcer, and two cultures of *M. ulcerans* from patients in east and west Africa who served as positive controls was extracted using the QIAamp DNA mini-kit (Qiagen, Valencia, CA). Briefly, mycobacterial pellets from the two cultured samples were resuspended in 180 μ L of buffer ATL (Qiagen) and 20 μ L of proteinase K was added. The mixture was incubated at 56°C overnight, 200 μ L of buffer AL (Qiagen) was added, the mixture was incubated at 70°C for 10 minutes, and 200 μ L of absolute ethanol (96–100%) was added. The mixture was then applied to the QIAamp minispin column (Qiagen) and centrifuged at 6,000 $\times g$ for 1 minute. The spin column was washed twice with buffers W1 and W2 (Qiagen) and the purified DNA was eluted with 200 μ L of elution buffer AE (Qiagen).

For the two Kenyan patient samples, tips of the swabs containing lesion exudate were cut, immersed in 400 μ L of phosphate-buffered saline, and 20 μ L of proteinase K, and 400 μ L of buffer AL (Qiagen) were added. The mixture was pulse vortexed, incubated at 56°C for 1 hour, and 400 μ L of absolute ethanol was added and mixed by pulse vortexing. The mixture was then applied to the QIAamp minispin column (Qiagen) and centrifuged at 6,000 $\times g$. The column was then washed with buffers W1 and W2 and DNA eluted with 200 μ L of elution buffer AE (Qiagen).

For real-time PCR, primers F1 (5'-ATT-GGT-GCC-GAT-CGA-GTT-G-3') and R1 (5'-TCG-CTT-TGG-CGC-GTAA-3') were used to amplify a region of insertion sequence (IS)2404, and probe FAM-5'-CACCACGCAGCATTCTTG CCGT-3'-TAMRA was used to detect the specific sequence, as described.⁵ IS2404 codes for an insertion sequence transposase found in multiple copies in the bacterial genome of *M. ulcerans*, recently found in several other less common mycobacteria,⁶ and is commonly used as a target sequence for *M. ulcerans* diagnosis.^{7,8} The reaction mixture contained 10 μ L of template DNA, each primer at a concentration of 0.4 μ M, 0.15 μ M of the probe, and TaqMan universal PCR master mixture (Applied Biosystems, Foster City, CA) in a total volume of 50 μ L. Detection of the PCR was carried out on an ABI prism 7500 sequence detection system (Applied Biosystems) under the following conditions: 1 cycle at 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute.

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FIGURE 1. Four pristine ulcers with surrounding mild hyper-pigmentation and-scaling, features characteristic of Buruli ulcer, on the right hip and thigh of patient FO. This figure appears in color at www.ajtmh.org.

For conventional PCR, DNA was amplified in a nested two-step reaction. The primary amplification was conducted in a 50- μ L reaction mixture containing 1 μ M each of primer pPG1 (5'-AGG-GCA-GCG-CGG-TGA-TAC-GG-3') and primer pPG2 (5'-CAG-TGG-ATT-GGT-GCC-GAT-CGA-G-3'),⁹ 2.5 units of Amplitaq Gold DNA Polymerase (Applied Biosystems), 200 μ M of each deoxyribonucleoside triphosphate and 1 \times PCR buffer (Applied Biosystems). The PCRs were conducted in a Gene Amp 9700 PCR system (Applied Biosystems) under the following conditions: denaturation at 94°C for 10 minutes, amplification for 45 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The primary product was kept at 4°C. A total of 0.2 μ L of the primary PCR product was used for a hot-start nested PCR using the same PCR master mixture as above and primers pGP3 (5'-GGC-GCA-GAT-CAA-CTT-CGC-GGT-3') and pGP4 (5'-CTG-CGT-GGT-GCT-TTA-CGC-GC-3'), with 25 cycles. A total of 5 μ L were loaded onto a 2% agarose gel stained with ethidium bromide and subjected to electrophoresis.⁹

The conventional PCR IS2404 amplicon from the sample from patient FO was then sequenced on an 3130xL ABI genetic analyzer using version 3.1 of the Big Dye terminator method (Applied Biosystems). The amplicon sequence from patient FO was compared against mycobacterial databases of the National Center for Biotechnology Information (Bethesda, MD) using the Basic Local Alignment Search Tool (BLAST).

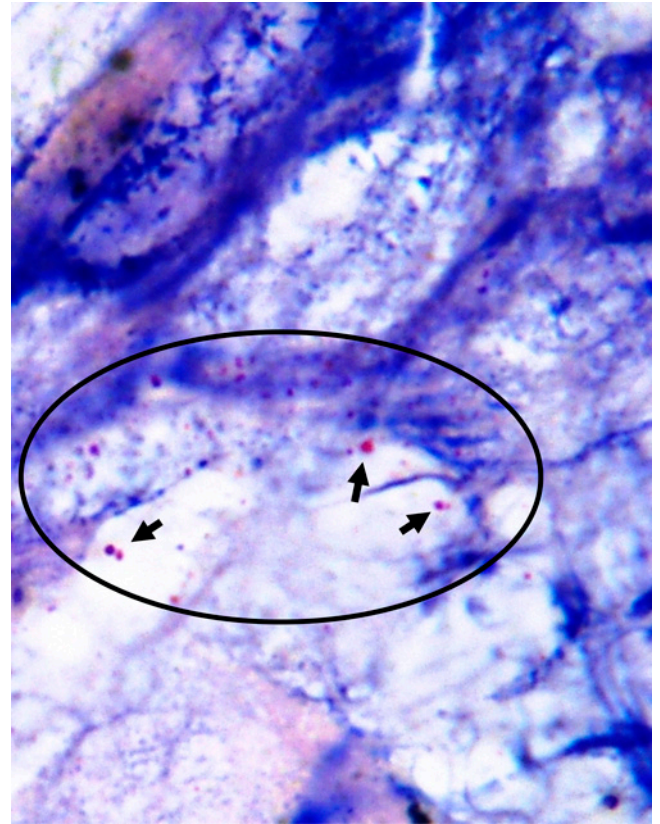


FIGURE 2. Ziehl-Neelsen staining of lesion smear of patient FO showing numerous clusters of pink-red acid-fast bacilli (AFB; **circle** and **arrows**). This figure appears in color at www.ajtmh.org.

Three IS2404 amplicons from the sample from patient FO and two positive controls (west and east African strains) were aligned against each other using multiple sequence comparison by log-expectation (MUSCLE) software version 3.6.^{10,11}

Real-time PCR of lesional material from patient FO using IS2404 primers with a specific probe showed amplification above the cycle threshold (Ct) value (Ct = 37.6), which supports identification as *M. ulcerans* (Figure 3). Late replication was consistent with a small amount of DNA. A west African *M. ulcerans* positive control sample also amplified and had lower Ct values of 15.3 and 15.9. A sample from a patient (JO) with a non-specific, AFB smear-negative ulcer showed no amplification.

Conventional PCR of lesional material from patient FO using IS2404 primers showed a product on electrophoresis of approximately 400 basepairs, which was similar to BU-positive control material and supported the diagnosis of BU (Figure 4). Analysis of the nonredundant nucleotide database at GenBank using BLAST showed that sequences of lesional material of patient FO with the highest identity were all IS2404 genes belonging to the reference strain *M. ulcerans* Agy99. Alignment analysis by MUSCLE of the IS2404 amplicon from patient FO and 2 *M. ulcerans* positive control samples, one each from east and west Africa, indicated a high degree of similarity among the three sequences, punctuated by small nucleotide variations in the same general regions (Figure 5).

To the best of our knowledge, this is the first report of a patient from Kenya presumed to have BU. Given the presence of BU in many west and central African counties, recent confir-

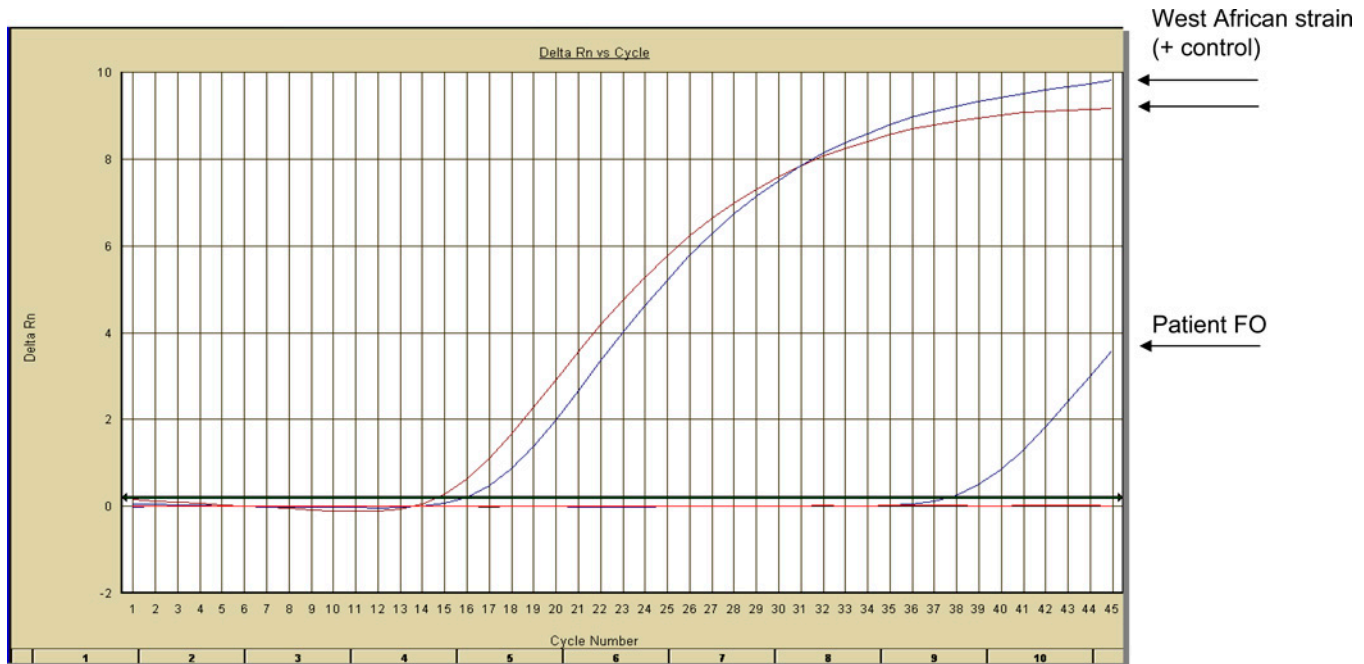


FIGURE 3. Real-time polymerase chain reaction amplification of an insertion sequence (IS)2404 gene fragment by using *Mycobacterium ulcerans* IS2404-specific primers and probe. Also shown are West African positive control strains (Ct 5.3 and 15.9) and Kenyan patient FO sample (Ct 37.6). This late cycle, compared with the positive control, was likely caused by relatively small amounts of *M. ulcerans* DNA. Lesion material from a second patient with an acid-fast bacilli smear-negative ulcer was not amplified. This figure appears in color at www.ajtmh.org.

mation of its presence in southern Sudan,² and a BU epidemic in nearby Uganda in the 1960s, BU in Kenya is not unexpected. Endemic foci of BU are most common near rural permanent wetlands in warm geographic regions, especially in areas prone to seasonal flooding. We speculate that BU is present to some degree in Kenya, especially in tropical low-lying areas such as Kisumu and the Lake Victoria region, but is not routinely considered in the differential diagnoses for skin ulcers.

Unlike tuberculosis and leprosy, BU is related to environmental factors and thus is considered noncommunicable. The most plausible mode of transmission is by skin trauma

at sites contaminated by *M. ulcerans*.¹² Our patient could not recall antecedent trauma at the lesion sites. The distribution of patients, even in highly endemic foci, is random, suggesting that each patient is exposed to some different environmental source. Studies in African populations suggest that risk factors include exposure to swamp water, swimming or wading in rivers, use of unprotected water sources for domestic purposes, such as our patient reported, low levels of schooling, and infection with human immunodeficiency virus.¹³⁻¹⁵

Specific insertion sequences for *M. ulcerans* that include IS2404 have been characterized and have identified the organism by PCR, although recent data indicate that several other less common mycobacteria may be positive for IS2404.^{6,8,16} In our patient, multiple pristine, undermined ulcers on the leg, a Ziehl-Neelsen stain of lesional material showing AFB, supportive real-time and conventional PCR outcomes using *M. ulcerans* IS2404 primers, IS2404 gene fragment homogeneity with *M. ulcerans* Agy99, and notable sequence alignment for the IS2404 PCR product against 2 control strains of *M. ulcerans* provided evidence that supported *M. ulcerans* as the cause of her ulcers. Because of the tenderness on palpation, we speculate that a hip radiograph would have shown bone lesions. Clinically, most other IS2404-positive pathogenic mycobacteria, including *M. marinum*, usually cause granulomatous skin lesions, unlike the contiguous coagulation necrosis caused by *M. ulcerans*.¹⁷ The shortcomings of our case workup included lack of culture, biopsy, and variable number of tandem repeats (VNTR) analysis, with the latter requiring biopsy or cultured organisms for definitive differentiation of *M. ulcerans* from other IS2404-positive mycobacteria.⁶

Using the MUSCLE program, we showed that IS2404 PCR product sequence alignment of lesional sample material from our patient and two control *M. ulcerans* samples had convincing homogeneity, along with scattered variations.

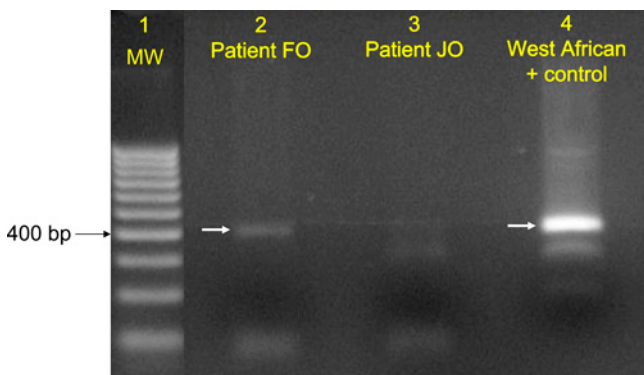


FIGURE 4. Electrophoresis of conventional polymerase chain reaction (PCR) products after amplification with *Mycobacterium ulcerans*-specific insertion sequence 2404 primers. Lane 1, molecular weight (MW) marker; lane 2, patient FO; lane 3, patient JO (non-specific, acid-fast bacilli smear negative ulcer); lane 4, *M. ulcerans* positive control. **Arrow** denotes PCR product of approximately 400 basepairs, consistent with *M. ulcerans* for lanes 1 (patient FO) and 4 (positive control), but absent in lane 3 (patient JO). This figure appears in color at www.ajtmh.org.

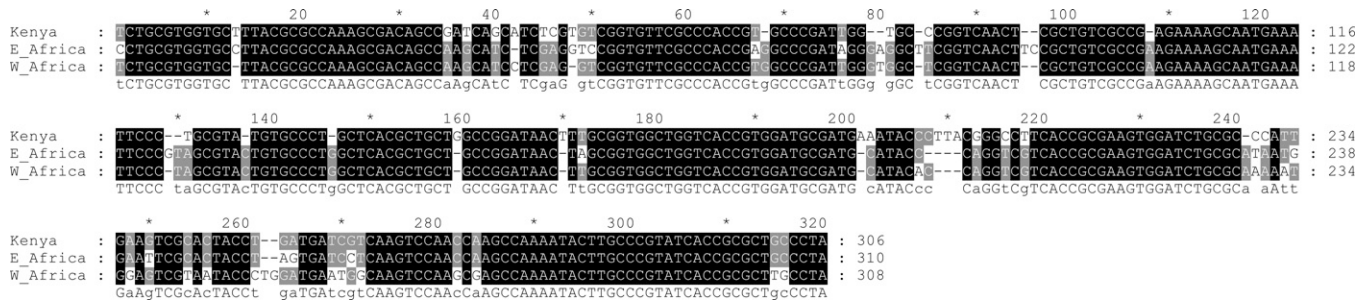


FIGURE 5. DNA sequence alignment of three amplicons after polymerase chain reaction for *Mycobacterium ulcerans* insertion sequence 2404, using MUSCLE software version 3.6. Kenya = patient FO; East Africa and West Africa = *M. ulcerans* positive control strains.

Notably, most of the variations occurred at the same locations, supporting species similarity. This finding was not unexpected because variations in IS2404 and VNTRs occur in African *M. ulcerans* isolates, especially when east and west African strains are compared.^{6,18} Despite notable sequence homology of our patient's IS2404 amplicon with *M. ulcerans* Agy99 and 2 control strains, we could not identify an exact match with any of the sequences on PubMed, which suggests a possible west Kenya variant.

A rapid reemergence of BU began in the early 1980s and is thought to be attributable to environmental factors such as deforestation, artificial topographic alterations (dams and irrigation systems), enlarging populations engaged in basic manual agriculture in wetlands, and possibly global climatic changes.¹⁹ The likely identification of BU in Kenya emphasizes the importance of considering BU in the differential diagnosis for any chronic ulcerative lesion in Africa, especially in areas characterized by wetlands.

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