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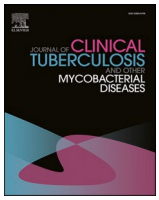

Pilot use of a mycolactone-specific lateral flow assay for Buruli ulcer: A case report from Japan

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ABSTRACT

Buruli ulcer, caused by *Mycobacterium (M.) ulcerans*, is a neglected tropical disease (NTD) characterized by necrosis of the cutaneous tissue, predominantly affecting the limbs. The pathogenesis of this disease is mainly attributed to mycolactone, a lipid toxin produced by *M. ulcerans*. Here, we report the case of a 7-year-old Japanese girl who presented with worsening ulceration on her left forearm, extending to the elbow, following antimicrobial treatment. To evaluate disease progression, we used a mycolactone-specific lateral flow assay. The test yielded positive results in the advancing necrotic area, aiding in determining the extent of necessary debridement. After undergoing two debridement surgeries and receiving 38 weeks of antimicrobial treatment followed by skin grafting, the patient achieved cure. Timely diagnosis is imperative in avoiding prolonged treatment, highlighting the importance of readily available diagnostic point-of-care tests for Buruli ulcer. Moreover, detection of mycolactone not only can serve as a diagnostic tool for Buruli ulcer but also enables prediction of lesion spread and assessment of cure.

1. Introduction

Buruli ulcer is a necrotizing cutaneous disease caused by *Mycobacterium (M.) ulcerans* and is one of the neglected tropical diseases (NTDs) [1]. According to the number of reported cases from 2010 to 2017, Japan had a total of 52 cases reported to the World Health Organization (WHO). This was the second highest following Australia in prevalence, excluding the African region [2].

Unlike other cutaneous non-tuberculous mycobacterial diseases where the growth of mycobacteria causes ulcerations, ulcerations in Buruli ulcer are caused by a lipid toxin called mycolactone, synthesized by *M. ulcerans*. In its host, major biological effects of mycolactone include cytotoxicity causing necrosis of subcutaneous adipose tissue and analgesic effects causing skin ulcers with limited pain [3]. Furthermore, it inhibits translocation of secretory and transmembrane proteins in the endoplasmic reticulum and suppresses the production of diverse cytokines and chemokines, thereby inducing localized immunosuppression [4]. These mechanisms define the distinctive histological characteristics of Buruli ulcer, featured by necrosis in dermal and subcutaneous tissues, minimal inflammatory cell infiltration, and granuloma formation at the ulcer's periphery [5,6].

Early diagnosis and treatment are critical in preventing prolonged treatment and avoiding disability due to Buruli ulcer. Confirmatory laboratory tests to diagnose Buruli ulcer include Ziehl–Neelsen staining,

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mycobacterial culture, histological examination, and polymerase chain reaction (PCR) test. However, these tests may not always be readily available in areas where Buruli ulcer are endemic. In Japan, general health facilities cannot perform the PCR test for Buruli ulcer. The typical timeline for diagnostic tests involves approximately 1–2 days for the Ziehl-Neelsen staining, at least 12 weeks for mycobacterial culture, and 1–3 weeks for histological examination. Samples of suspected cases are then sent to the Leprosy Research Center at the National Institute of Infectious Diseases in Tokyo, which serves as the national reference center for the disease. Here, the confirmatory PCR test is performed, and results are provided within 3–7 days. Consequently, the overall process of diagnosing Buruli ulcer in Japan can be time-consuming, leading to delayed diagnosis.

Efforts to develop point-of-care (POC) tests for detecting mycolactone are ongoing, aiming at rapid diagnosis of Buruli ulcer in field settings and general clinical facilities, as outlined in the WHO Target Product Profile for Buruli ulcer [7]. Unlike PCR, which analyzes DNA and cannot differentiate between live and dead bacilli, detecting mycolactone can be used not only for diagnosis but also to assess disease progression and cure. In this report, we present a case of Buruli ulcer in a 7-year-old female in Japan. We employed a prototype lateral flow assay for mycolactone detection, currently under development, to analyze the presence of mycolactone at different surgical sites of the affected lesion to assess disease progression.

2. Prototype lateral flow assay for mycolactone detection

The lateral flow assay in this case report employs mycolactone-specific monoclonal antibodies and biotinylated mycolactone probes first used in a previously described ELISA [8] for the detection of mycolactone. Reagents were used for the design of a competition lateral flow assay ultimately intended as POC for resource-limited settings. This lateral flow assay is currently being tested for efficacy and is not yet a commercial product. Wound exudates collected with a swab were used as samples, followed by a concentration step. Mycolactone is visualized by the lateral flow assay within two hours using a mouse monoclonal antibody specific for mycolactone as antigen capturing reagent. Magnetic gold nanoshells covalently conjugated to the antibody enable the immunologic concentration of the targeted analyte. If the test sample contains no mycolactone, the conjugate binds to a biotinylated synthetic mycolactone probe producing a dark test line. Mycolactone present in the test sample saturates the binding sites of the anti-mycolactone antibody, preventing capture of the mycolactone probe at the test line. Consequently, the test line intensity is inversely proportional to the concentration of mycolactone in the test sample. The control line contains an anti-mouse antibody as control for proper sample flow and reagent functionality. The quantification process using a benchtop reader measures the intensity of the test line and the ratio between the test and control lines (T/C), with the T/C ratio providing quantitative results. Detailed documentation of the assay is available elsewhere [9]. This test is used in a clinical evaluation study in Japan and is under investigation along with other international study sites. Ethical approval for the use of this lateral flow assay on a patient was obtained from the ethics committees of Tonami General Hospital (No. 2022010).

3. Case

In November 2021, a 7-year-old Japanese girl reported being bitten on her left forearm by an insect. She was a resident of Toyama Prefecture, a west coastal region of Japan, with no history of international travel. By the end of the same month, the initial red lesion got covered with a black crust, which gradually enlarged. In January of the following year, she visited our hospital (Tonami General Hospital, Toyama Prefecture, Japan), where she was first diagnosed with a black crust resulting from an insect bite. Subsequently, the crust became ulcerated, and edema was observed around the lesion. She was taken to a near-by hospital by her parents. Secondary infection was suspected, and she was put on cefazolin 80 mg/kg/day intravenously for five days. However, there was no improvement, prompting her admission to our hospital in February.

Upon physical examination at admission, the patient was afebrile with no lymphadenopathy. A dry ulcer of approximately 1 cm was observed on the patient's left forearm, with a white and firm base. Redness, edema, and warmth were observed around the ulcer and throughout the entire forearm, accompanied by mild tenderness but an absence of spontaneous pain. There were no limitations in the movement of the patient's elbow or wrist joints.

Magnetic Resonance Imaging of the left forearm showed subcutaneous edema, with no abnormal findings in the muscles, bones, or joints. A skin punch biopsy was performed at the ulcer's border the following day after admission. Histological examination results at our hospital, which came nine days later, revealed necrosis in the dermis and subcutaneous tissue, with no inflammatory cell infiltration, granulomas, or atypical cells. A stamp sample from the entire ulcer base, along with histological examination of the subcutaneous tissue, identified the presence of acid-fast bacilli on Ziehl–Neelsen staining, which led to the suspicion of Buruli ulcer. The skin punch biopsy specimens were cultured for mycobacteria at a commercial laboratory, BML Inc. Initially, cultures were incubated at 37 °C on 2 % Ogawa medium. Following the identification of mycobacterial infection by the histological examination at ninth day, the incubation temperature was adjusted to 30 °C. After eight weeks of culture, five colonies of mycobacteria were observed. BML Inc. did not offer subsequent identification testing by PCR test for *M. ulcerans* or genome sequencing. Therefore, these procedures could not be performed. Although shipping of specimens to the Leprosy Research Center, National Institute of Infectious Diseases for PCR testing was considered, the scarcity of the remaining specimens prevented this. It was instead planned for the time of the first debridement.

Following the initiation of antimycobacterial drugs including rifampicin (RFP) 10 mg/kg per body weight daily, clarithromycin (CAM) 10 mg/kg per body weight twice daily, and tosufloxacin (TFLX) 4.1 mg/kg per body weight twice daily, the patient was discharged on Day 7 with reduced redness and swelling in the left forearm (Fig. 1a). The area with redness on the forearm deepened in color to dark red during the second week post-initiation of antimycobacterial drugs, and the ulcer area expanded during the fourth week (Fig. 1b).

On Day 34 post-initiation of antimycobacterial drugs, the patient underwent the first debridement under general anesthesia. Histological examination of the excised skin sample showed findings similar to those of the initial punch biopsy (Fig. 2a, b). The skin sample from the excised marginal area was shipped to the Leprosy Research Center, National Institute of Infectious Diseases. PCR conducted at the center confirmed the presence of the IS*2404* gene of *M. ulcerans*, confirming the diagnosis of Buruli ulcer [10]. However, the culture yielded a negative result.

One week after the first surgery, necrosis developed in the unexcised skin within the dark red and edematous area (Fig. 1c). A second debridement surgery was performed on Day 118. Prior to the surgical intervention, swab samples were taken from four different lesion sites as shown in Fig. 1c. PCR testing and a mycolactone-specific lateral flow assay were performed on each swab at the Leprosy Research Center, National Institute of Infectious Diseases. All swabs tested positive by PCR (Fig. 2e), while only sample #4 from the periphery of the necrotic site was positive with the lateral flow assay (Fig. 3). The histological examination of the second excised dermal sample showed granuloma formation in the subcutaneous tissue and a moderate inflammatory cell infiltrate, predominantly lymphocytes, surrounding the granulomas. On the other hand, there was no inflammatory cell infiltrate adjacent to the necrotic dermis and subcutaneous tissue where sample #4 was taken (Fig. 2c). At the same site, there was no detectable presence of acid-fast bacilli in the subcutaneous tissue (Fig. 2d). Once again, the culture result was negative.

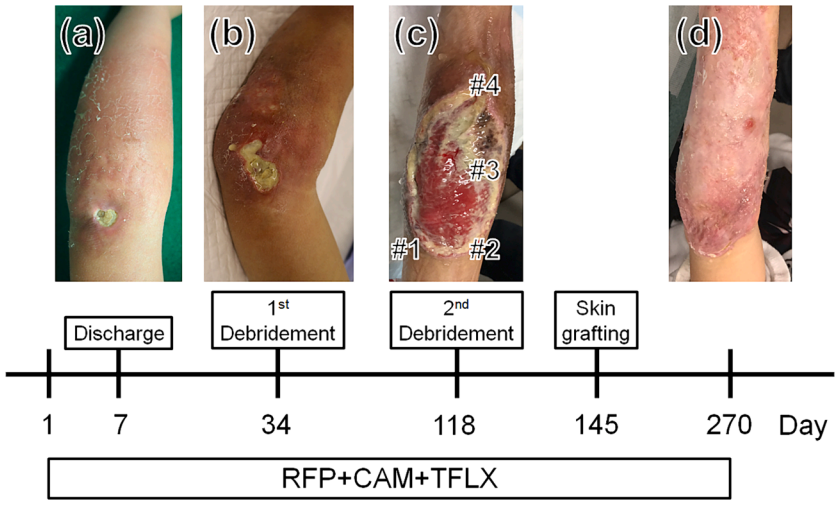

Fig. 1. Clinical course and symptomatic changes of Buruli ulcer on the left forearm. (a) At the time of discharge, the ulcer measured approximately 1 cm, accompanied by redness and edema around the ulcer, extending to the forearm. (b) Four weeks after initiation of antimicrobials, the ulcer enlarged, and the area with redness turned dark red. (c) Following the first debridement surgery, skin necrosis, which was previously dark red and edematous at the peripherally ulcerated with thick, brownish necrotic tissue. # Numbers indicate the sites where swab samples were obtained for the mycolactone-specific lateral flow assay. (d) At the end of treatment. After confirming the epithelialization of the grafted skin, antimycobacterial drugs were terminated. RFP: rifampicin, CAM: clarithromycin, TFLX: tosufloxacin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

On Day 145, the patient underwent segmental skin grafting. Following the complete epithelialization in the grafted skin without recurrence of Buruli ulcer (Fig. 1d), the 38-week course of antimycobacterial drugs including RFP, CAM, and TFLX was terminated. The patient experienced no adverse drug reactions during treatment.

4. Discussion

We presented the first confirmed case of Buruli ulcer in Toyama Prefecture, Japan, and the first case in which the persistent presence of mycolactone was successfully assessed using a lateral flow assay prototype. The testing was conducted at multiple points on the ulcer, with a positive result exclusively in the peripheral area where skin necrosis had progressed following surgery.

The patient developed an enlarged skin ulcer area four weeks after initiating antimycobacterial drugs, followed by progressive skin necrosis in the dark red and edematous area after the first surgery. These manifestations met the clinical diagnostic criteria for a paradoxical reaction [11], characterized by disease exacerbation during or after antibiotic treatment [12]. This is known to typically occur due to the recovery of inflammatory responses following reduced mycolactone synthesis from treatment. To distinguish the paradoxical reaction from ongoing infection, we conducted a histological examination following the first debridement. This showed the absence of inflammatory cell infiltrate, and although acid-fast bacilli were present, culture was negative. Despite the unavailability of a 16S rRNA assay [12] to detect viable bacteria, the histological findings indicated immunosuppression and necrosis induced by mycolactone, rather than paradoxical reactions.

Ethical approval for the mycolactone-specific lateral flow assay study at our hospital was obtained in May 2022, allowing us to apply the test during the second debridement surgery which happened in June. It was interesting to observe a positive result exclusively in the sample taken from the peripheral area exhibiting necrosis, while the samples from other areas yielded negative results. Additionally, the discordance between the PCR and the lateral flow assay results was interesting as it suggested that PCR is not suitable for assessing disease activity. This was further supported by the histological findings, as the absence of inflammatory infiltrate – indicative of the presence of mycolactone – was only observed at the same site (#4).

In a cohort of patients from Australia, edematous lesions have been identified as a significant risk factor for paradoxical reactions [11]. In our case, the presence of edematous lesions necessitated a careful distinction from a paradoxical reaction. However, our results ruled out a paradoxical reaction, revealing findings attributed to the effects of mycolactone. A report of mass spectrometry analysis of Buruli ulcer described that the distribution of mycolactone varied depending on the type of skin lesion. Specifically, mycolactone was detected over a wide area leading to severe ulcer formation in edematous lesions, even at lower concentrations [13]. These findings suggest that some cases previously diagnosed as paradoxical reactions in edematous lesions could have been, in fact, due to residual mycolactone. Mycolactone is diffusible, and its clearance from human skin tissues is known to take long, ranging from days to weeks [13]. Therefore, this increases the importance of mycolactone as a biomarker for Buruli ulcer, not only for diagnosis but also as a predictive test to identify the site of lesion spread.

Our patient exhibited symptoms in November, consistent with the seasonal trend observed in Japan. Previous studies have reported that approximately 80 % of Buruli ulcer cases in Japan are diagnosed in autumn and winter between September and February, with few cases in summer between July and August [14]. However, similar to other countries endemic with Buruli ulcer, the transmission route of the disease in Japan remains unclear, making it difficult to determine the reason behind this seasonal trend. Occurrence of Buruli ulcer is often associated with water bodies, as many patients diagnosed with the disease tend to live near them [14]. In our case, the patient's house was located in an area with many rice fields, aligning with the patterns.

In Japan, treatment of Buruli ulcer with a three-drug regimen of RFP, CAM, and levofloxacin (LVFX) in adults, and TFLX as a substitute for LVFX in children, has been reported to achieve higher cure rates without surgery compared to other antibiotic regimens [15]. TFLX was chosen due to its pediatric insurance coverage, although data on its effectiveness against *M. ulcerans* are lacking [16]. Our patient underwent a prolonged course of antimycobacterial drug treatment, exceeding the WHO recommended duration of 8 weeks [17]. Treatment duration in Japan varies widely, ranging from 2 to 48 weeks [15], with no standardized period established. Consequently, treatment often continues until clinical cure, potentially leading to overtreatment [16]. This practice has been driven by the absence of feasible measures to identify cure and confidently determine when to cease treatment. It is advisable to shorten the treatment period of cases, and the mycolactone-specific

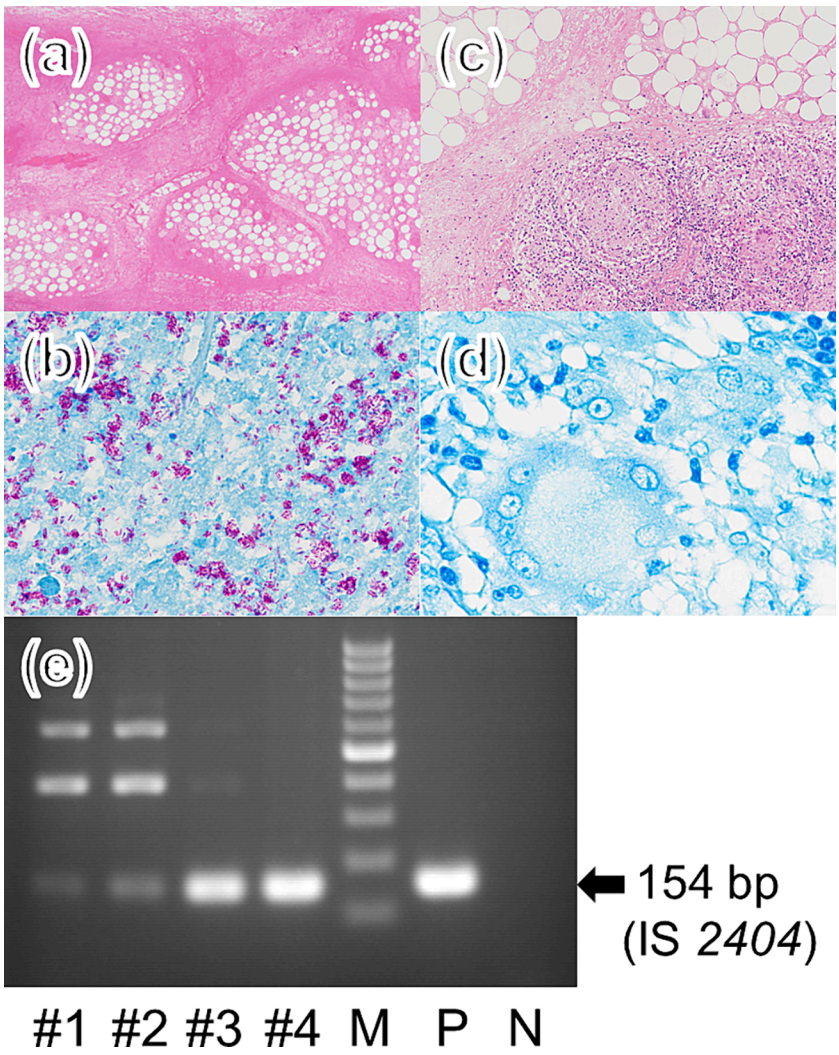

Fig. 2. Histological findings and PCR testing. At the first debridement surgery, (a) Necrosis of the dermis and subcutaneous tissue with an absence of inflammatory cell infiltrate (hematoxylin-eosin staining, original magnification × 100), (b) Numerous clusters of acid-fast bacilli in the subcutaneous tissue (Ziehl–Neelsen staining, original magnification × 400). At the second debridement surgery, (c) Necrosis of dermis and subcutaneous tissue with an absence of inflammatory cells. Adjacent to it, a granuloma formation in the subcutaneous tissue with a moderate inflammatory cell infiltrate, predominantly lymphocytes, surrounding the granulomas (hematoxylin-eosin staining, original magnification × 200). (d) No acid-fast bacilli in the subcutaneous tissue (Ziehl–Neelsen staining, original magnification × 1000). (e) PCR amplification of IS*2404* gene of *M. ulcerans* in each swab. M, 100 bp ladder marker; P, positive control; N, negative control.

lateral flow assay can assist in assessing cure and deciding when to discontinue the use of antimicrobial drugs.

Given the limited scope of this being a single case report, it is essential to evaluate the performance of the mycolactone-specific lateral flow assay in a larger cohort.

Our case demonstrated that the mycolactone-specific lateral flow assay was useful in confirming the presence of mycolactone and its relationship with the clinical manifestations. This emphasizes the importance of innovative diagnostic tools in improving patient outcomes for Buruli ulcer. Further study with more patients is expected to provide additional validation of the efficacy and reliability of the mycolactone-specific lateral flow assay in diverse clinical settings and geographic locations.

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Informed consent

This study was reviewed and approved by the ethics committees at the Tonami General Hospital (No. 2022010) and the National Institute of Infectious Diseases (No. 1396), as complying with the Ethical Guidelines for Medical and Health Research Involving Human Subjects established in Japan. The patient's parents provided written informed consent allowing the use of the patient data for scientific research and we anonymized all identifiable details to protect privacy.

CRediT authorship contribution statement

Yasuhisa Sakakibara: Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Michio Konishi:** Investigation. **Teruo Ueno:** Investigation. **Chiaki Murase:** Investigation. **Yuji Miyamoto:** Investigation. **Manabu Ato:** Investigation. **Dziedzom K. de Souza:** Writing – review & editing, Conceptualization. **Marco Biamonte:** Supervision, Conceptualization. **Gerd Pluschke:** Supervision, Conceptualization. **Rie R. Yotsu:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization.

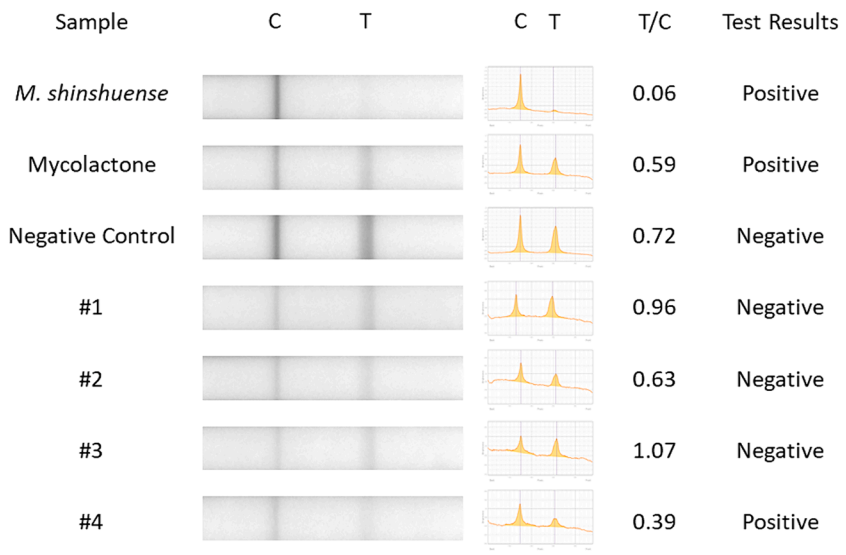

Fig. 3. Mycolactone-specific lateral flow assay results. The control line verifies proper sample flow and reagent function by its intensity. The test line detects mycolactone presence through a competitive immunoassay, with a weaker intensity indicating a higher concentration of mycolactone. C, control line; T, test line; T/C, T line peak value divided by C line peak value; *M. shinshuense*, *M. ulcerans* subsp. *shinshuense*. Sample #4 tested positive, indicated by a lower value compared to the positive control, mycolactone, at T/C. The remaining samples #1,2,3 tested negative as indicated by higher values than the positive control.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dziedzom K. de Souza, Marco Biamonte, Gerd Pluschke, Rie R. Yotsu reports financial support was provided by Global Health Innovative Technology (GHIT) Fund (G2020-202). Yuji Miyamoto, Manabu Ato reports financial support was provided by Japan Agency for Medical Research and Development. Rie Yotsu is one of the guest editors for the Special Issue on Buruli Ulcer in the Journal of Clinical Tuberculosis and Other Mycobacterial Diseases. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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