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Isolation of *Mycobacterium ulcerans* from Swab and Fine-Needle-Aspiration Specimens▽

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For cultivation of *Mycobacterium ulcerans* from clinical specimens, we optimized the release of bacteria from swabs, as well as decontamination and cultivation on supplemented medium. Nevertheless, the proportions of positive cultures, 41.7% (5/12) for fine-needle-aspiration (FNA) samples and 43.8% (49/112) for swab samples, were lower than those we have previously observed for excised tissue specimens.

Buruli ulcer (BU), a necrotizing disease caused by the environmental mycobacterium *Mycobacterium ulcerans*, is the third most important mycobacteriosis globally, after tuberculosis and leprosy (1, 9, 11). Laboratory diagnosis of BU is performed by the microscopic detection of acid-fast bacilli, cultivation of the pathogen from clinical specimens, identification of characteristic histopathological changes, and detection of *M. ulcerans*-specific DNA by PCR (10). The isolation of *M. ulcerans* from clinical samples is a slow and difficult process due to many factors that have been previously well documented (9, 10). Traditionally, BU case management has been wide surgical excision of BU lesions to remove infected tissues followed by skin grafting. Isolation of the causative agent therefore relied primarily on tissue samples from the surgical interventions (4, 7, 12). Hence, we previously optimized *M. ulcerans* cultivation from such tissue specimens and achieved recovery rates of >75% (7, 12). However, in 2006, the World Health Organization (WHO) introduced a combination of rifampin and streptomycin or amikacin for 8 weeks as a first-line treatment for all forms of the active disease, and this has been reported to reduce the rate of recurrence. Surgery is therefore done to remove necrotic tissue and correct deformities after antibiotic treatment (3, 6). As a result, punch biopsy specimens are now the only source of tissue samples from untreated patients for the isolation of *M. ulcerans*. It is recommended that this invasive sampling procedure be used only when reconfirmation of clinical diagnosis from swabs and/or fine-needle aspirations (FNAs) has failed. Therefore, suitability of specimens obtained by less-invasive procedures, such as FNA from nonulcerative lesions and swabs from the undermined edges of ulcerative lesions for primary isolation of *M. ulcerans*, needs to be evaluated (5, 8).

Swab and FNA specimens were collected from Buruli ulcer patients presenting with active ulcerative and nonulcerative lesions, respectively. All patients included in this study were diagnosed according to the clinical definition of the WHO (10), and clinical diagnosis was confirmed by a positive IS*2404* PCR laboratory test (7). To prevent overgrowth by other bacteria, swabs were collected in a 15-ml conical tube (BD) containing 5 ml semisolid transport medium (4, 12) with 2% PANTA Plus (Becton Dickinson, Franklin Lakes, NY), to cover the entire tip of the swab. FNA specimens were immediately drained into a 1.5-ml sterile screw-cap tube containing 500 μl liquid transport medium with the same percentage of antibiotics (10, 12). Within 24 h, samples (on ice) were transported from the participating health facilities of the Ga District (Amasaman District Hospital, Obom Health Centre, and Kojo Ashong Community Clinic) to the cultivation facility of the Noguchi Memorial Institute for Medical Research (Legon, Ghana) for analysis. Ethical clearance for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research. One or two swab specimens were taken from the undermined edges of ulcerative lesions. In the latter case, the two samples were pooled together in 2 ml of phosphate-buffered saline (PBS) and vortexed for 2 min in 50-ml tubes containing glass beads (3-mm diameter; Merck, Germany) to disperse off as much as possible all bacteria attached to the swab into the PBS. Five hundred microliters of bacterial suspension was used for DNA extraction with the QIAamp DNA minikit (Qiagen, Hilden, Germany). IS*2404* PCR analysis was performed as described previously (7).

We evaluated three different decontamination procedures for the efficiency of decontamination, as well as for *M. ulcerans* recovery rates. Aliquots of suspensions prepared from 20 swab specimens of 20 PCR-positive patients were decontaminated in parallel using the Petroff method (12), the oxalic acid method (12), or a double decontamination method (the oxalic acid method followed by the Petroff method). After decontamination, specimens were inoculated onto two slants of Lowenstein-Jensen (LJ) medium. With either Petroff or oxalic acid single decontamination, 10/20 (50%) of the specimens were culture positive (Table 1). Contamination was observed with only one (5%) or two (10%) of the cultures after oxalic acid or Petroff decontamination, respectively. After double decontamination, 19/20 (95%) of the samples were culture negative; *M. ulcerans* growth was recorded for only one sample (Table 1). This find-

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TABLE 1. Result obtained with the different decontamination procedures that were evaluated in this study[a]

Decontamination method	No. (%) of cultures		
	Culture positive (n = 20)	No bacterial growth (n = 20)	Contaminated (n = 20)
Oxalic acid method	10 (50)	9 (45)	1 (5)
Petroff method	10 (50)	8 (40)	2 (10)
Double decontamination	1 (5)	19 (95)	0 (0)

[a] A culture was considered positive if at least one tube gave an isolate that was confirmed as *M. ulcerans* and contaminated if all tubes inoculated were overgrown by other bacteria.

ing agrees with the result of previous work on optimization of decontamination procedures for the isolation of *M. ulcerans* from tissue samples in our laboratory (12). We therefore chose the oxalic acid decontamination method for subsequent analysis because we find it convenient, as we are using it in other mycobacterial isolation systems.

Using the oxalic acid decontamination method, we analyzed in a next step swabbed exudates from 112 ulcerative lesions of 98 patients and 12 FNA specimens from nonulcerative lesions of 12 patients. All lesions were IS*2404* PCR positive. Sixty-seven percent (75/112) of the swab samples were microscopy positive for acid-fast bacilli after concentration of the bacterial suspension. A comparable positivity rate, 66.7% (8/12), was observed with smears prepared from the FNA samples (Fig. 1). Two slants of LJ medium, one supplemented with 2% PANTA Plus and the other containing no antibiotic supplement, were inoculated per sample. Fifty-four of 124 (43.5%) of the samples yielded positive culture, 4 (3.2%) became contaminated, and 66 (53.3%) yielded no growth. Of the 54 positives, 5 were FNA samples and 49 were swab samples, giving comparable isolation rates for the two types of specimens, i.e., 41.7% (5/12) for FNA and 43.8% (49/112) for swabs. Of the 54 culture-positive samples 50 (92.6%) grew on our selective LJ medium supplemented in-house with PANTA Plus, while 34 (66.7%) grew on standard LJ medium ($P < 0.001$). In view of the apparent low recorded sensitivity of primary culture of *M. ulcerans* from swabbed exudates and FNA compared to PCR and microscopy, we recommend that PCR and microscopy should first be performed on specimens received in the laboratory. Culture then should be done only when either both or PCR test positive to obtain isolates for further analysis, including microepidemiological studies.

As part of our laboratory diagnostic services for the Ghana Health Service, we routinely receive swab samples from clinical diagnosed Buruli ulcer patients for confirmation of clinical diagnosis by IS*2404* PCR. For this purpose, samples are generally shipped as dry swabs; they usually reach the laboratory between 7 days and 1 month after collection. Of 9 of such IS*2404* PCR-positive samples cultured after oxalic acid decontamination, 3 (33.3%) were culture positive and 6 (66.7%) yielded no bacterial growth. This preliminary analysis indicates that dry samples routinely used for PCR-based reconfirmation of clinical diagnosis may also be suitable for culture-based surveillance for the potential emergence of antibiotic-resistant strains in remote areas of Buruli ulcer endemicity.

The diagnostic value of FNA specimens is well documented with respect to PCR and microscopy (2, 5, 8); however, the ability to isolate *M. ulcerans* from FNA samples is crucial with regard to the current mode of treatment. An important goal of Buruli ulcer surveillance and control strategies is early case detection. It is therefore envisaged that FNA sampling of nonulcerative lesions will gain increasing importance as a diagnostic approach. At the same time, the availability of *M. ulcerans* isolates will be paramount for surveillance of drug susceptibility, analysis of recurrences, and molecular epidemiological studies. Our results demonstrate that *M. ulcerans* isolates can be obtained at reasonable frequency from FNA samples when sample collection and transport are optimal. However, culture positivity rates observed in this report for FNA and swab samples were lower than our previously reported rate (>75%) for tissue specimens (12). Due to the apparent lower sensitivity of isolation of *M. ulcerans* from swab and FNA specimens as a result of the amount of collected specimen by these two sampling procedures, one is tempted to advocate punch biopsy as a more appropriate sampling procedure. However, we are of the opinion that due to the invasiveness of punch biopsy, a new study assessing the improvement in isolation rate when the number of swabbed samples taken is increased will be needed. In this analysis, at most two swabs were collected per lesion; it may therefore be possible to further increase the culture positivity rate by increasing the surface area sampled per swab and/or by increasing the number of swabs collected per lesion. While Eddyani et al. (5) used the double decontamination procedure to successfully solve the problem of contaminations associated with primary cultivation of *M. ulcerans*, it was too harsh in our hands; in contrast, single decontamination of swab samples yielded satisfactory results.

In conclusion, our results prove that noninvasive sampling procedures such as FNA and swabbing are useful not only for case confirmation by PCR but also for primary culture of *M. ulcerans*. This finding, especially in the case of FNA, is very

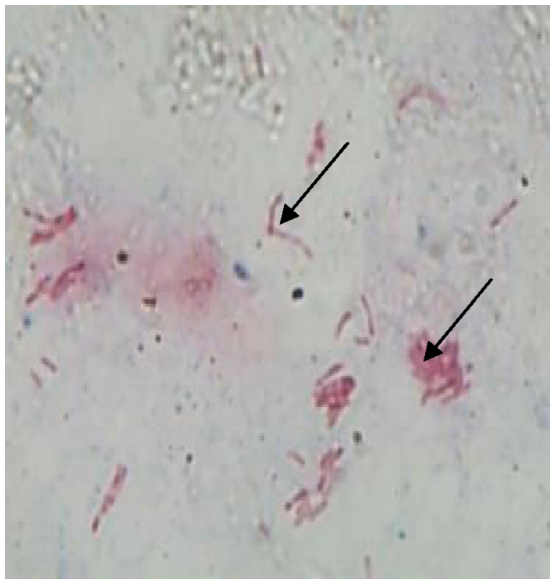

FIG. 1. Laboratory diagnosis of preulcerative Buruli ulcer lesion by Ziehl-Neelsen staining of a smear prepared from an FNA sample. The black arrows point to acid-fast bacilli.

important, as it allows the raising of isolates even from early cases, which will be beneficial for addressing a number of biological and epidemiological questions. This is very crucial in the case of *M. ulcerans*, for which current knowledge is very scant. Some of the questions that need to be addressed using strain collections are the mode of transmission and drug susceptibility pattern analysis.

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