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Evaluation of Decontamination Methods and Growth Media for Primary Isolation of *Mycobacterium ulcerans* from Surgical Specimens

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We evaluated four decontamination methods and one nondecontamination procedure in combination with four egg-based media for the primary isolation of *Mycobacterium ulcerans* from tissue specimens. With mycobacterial recovery and contamination rates of 75.6 and 2.4%, respectively, the combination of the oxalic acid decontamination method with Lowenstein-Jensen medium supplemented with glycerol yielded the best results.

At this time, laboratory diagnosis of Buruli ulcer (6) caused by *Mycobacterium ulcerans* relies on the detection of acid-fast bacilli in stained smears, isolation of the pathogen from infected tissue or tissue exudates by culture, identification of characteristic histopathological changes, and/or detection of mycobacterial DNA by IS*2404* PCR. A number of studies have indicated that the sensitivity of culturing the slow-growing *M. ulcerans* is <40% compared with IS*2404* PCR (1, 7, 8). The purpose of this study was to optimize the cultivation procedure of *M. ulcerans* for surgical specimens.

Tissue specimens were collected from patients suspected to have active Buruli ulcers according to the clinical definition of the World Health Organization (6). The specimens were collected from patients at the Amasaman Health Centre in the Ga district of Ghana between September 2002 and April 2003. Cultures were set up with a total of 41 surgical specimens collected from 36 patients. Patients usually sought medical treatment late, as more than half of them presented with ulcerative lesions. Care was taken not to contaminate the excised specimens, which were stored at 4°C in 7 ml of modified Dubos transport medium supplemented with 10% oleic acid-albumin-dextrose-catalase (KC Biologicals, Lenexa, Kans.), 2% PANTA Plus (Becton Dickinson, Franklin Lakes, N.Y.), and 0.5% agar (Difco). All samples were processed within 1 week after surgical excision. Specimens were cut into smaller pieces, homogenized, and suspended in 8 ml of Dulbecco's phosphate-buffered saline. One hundred microliters of the resulting suspension was inoculated directly, i.e., without prior decontamination, onto selective Lowenstein-Jensen (LJ) medium supplemented with 0.75% glycerol and 2% PANTA Plus. The remaining suspension was divided equally into four aliquots (about 2 ml in volume). Aliquots were decontaminated by the Petroff (P) (2), reversed Petroff (RP) (5), *N*-acetyl-L-cysteine (NALC) (2), or oxalic acid method (OA) (5). After decontamination, the homogenates were concentrated by centrifugation, and 100-μl aliquots of the 1-ml suspensions were inoculated in duplicate on the four solid media (9): LJ supplemented with 0.75% glycerol (LJG) or 0.5% pyruvate (LJP) and Ogawa medium supplemented with 1.3% glycerol (OG) or 0.5% pyruvate (OP). Inoculated tubes were incubated at 33°C, and the number of tubes that yielded *M. ulcerans*, mycobacterial colony count per tube, and length of time before visible colonies appeared were determined. All suspected *M. ulcerans* isolates were confirmed by sequencing the first 500 bp of the 16S rRNA gene using the MicroSeq 500 16S rDNA Bacterial Sequencing kit (PE Applied Biosystems) as described earlier (4).

LJG gave the best ($P < 0.001$) overall mycobacterial recovery rate when results obtained with the different decontamination methods used were pooled together (Table 1). It supported the growth of *M. ulcerans* from all 32 specimens from which a positive culture could be obtained by any of the conditions tested ($n = 41$). When the results of the four different media used were pooled together ($n = 144$ tubes per decontamination method), 4 (2.4%), 5 (3%), 24 (14.6%), and 25 (15.2%) tubes with OA, RP, P, and NALC, respectively, were found to be contaminated (Table 2). In combination with the best performing medium, LJG, the recovery rates were 75.6, 68.3, 65.8, and 63.4% for OA, P, RP, and NALC, respectively. Of the 41 culture tubes containing LJG supplemented with PANTA Plus and inoculated with noncontaminated samples, 25 (61%) grew *M. ulcerans*, 12 (29.2%) became contaminated, and 4 tubes (9.7%) showed no growth. Macroscopically detectable *M. ulcerans* colonies appeared faster ($P < 0.05$) with the nondecontamination method (6 weeks) than with the four decontamination procedures (median time with LJG, 8 weeks),

TABLE 1. Effect of the growth medium on the recovery of *M. ulcerans*

Medium[a]	No. of culture-positive samples ($n = 164$) (%)	Mean CFU/culture tube	Time until macroscopic growth[b]
LJG	112 (68.3)	215	5–14 (7.4)
LJP	78 (47.6)	100	6–13 (9.4)
OG	88 (53.6)	145	8–19 (12.5)
OP	52 (31.7)	80	8–18 (11.8)

[a] Results with the four different decontamination methods were pooled together.
[b] Length of time (in weeks) until macroscopic growth was observed. The range is given. The mean time is shown in parentheses.

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TABLE 2. Effect of the decontamination method on the recovery of *M. ulcerans*

Decontamination method[a]	No. of culture-positive samples (n = 164) (%)	No. of cultures with contamination (%)
P	81 (49.4)	24 (14.6)
RP	86 (53.8)	5 (3.0)
NALC	78 (47.6)	25 (15.2)
OA	86 (53.8)	4 (2.4)

[a] Results with the four different media were pooled together.

and the mean number of mycobacterial CFU obtained by the nondecontamination method was also found to be slightly higher than those obtained by the decontamination procedures. However, due to the high contamination rate, the number of positive cultures was slightly lower (odds ratio of 2.7; 95% confidence interval, 0.8 to 8.9) than that with the best performing decontamination method, OA in combination with LJG.

Of 41 samples analyzed, 32 (78%) were positive for acid-fast bacilli by microscopy after Ziehl-Neelsen staining (1), and the same number were culture positive (31 of these 32 samples were positive by the best performing cultivation methodology, LJG in combination with OA). Altogether, pure cultures were obtained from 30 of the 41 samples. Three of the culture-negative samples were positive by microscopy (for one sample, all culture tubes gave rise to nonmycobacterial overgrowth), and three of the microscopy-negative samples were culture positive. Of the six culture- and microscopy-negative samples, only one was positive by IS*2404* PCR (3, 8). We assume that the lesions from which the remaining five samples had been collected were not related to Buruli ulcer. Only slight differences in the performance of microscopy and culture by the type of lesion were observed (Table 3). Culture positivity was slightly higher with ulcerative lesions than with nodules and plaque lesions (86 versus 70%); however, this was not statistically significant.

TABLE 3. Performance of diagnostic microbiology by type of Burundi ulcer lesion

Disease stage	No. of microscopy-positive specimens[a]	No. of culture-positive specimens (all methods)[a]
Nodules and plaques	16/20 (80)	14/20 (70)
Ulcerative lesions	16/21 (76)	18/21 (86)
Total	32/41 (78)	32/41 (78)

[a] The number of microscopy- or culture-positive specimens to the number of total specimens is shown. The percentage is shown in parentheses.

In the present study, we used strict sampling procedures. (i) Great care was taken to avoid exogenous contamination when recovering samples from the surgically excised tissue. (ii) The collected tissue samples were immediately suspended in a medium containing antibiotics, kept refrigerated, and transported on ice. (iii) Samples were processed within 7 days after excision in a cultivation facility in Ghana. Adherence to these strict rules resulted in a comparatively low contamination rate; only one nodule specimen was lost due to bacterial overgrowth of all culture tubes.

We conclude that successful primary isolation of *M. ulcerans* depends on the emphasis put on optimum sampling procedures and specimen handling prior to cultivation. The direct inoculation of specimen onto a selective LJG medium containing PANTA Plus and the use of the OA decontamination method in combination with LJG medium may further increase recovery rates, particularly for specimens with a low mycobacterial load, and reduce the time before recovery of *M. ulcerans*.

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