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Application of Oxidation-Reduction Assay for Monitoring Treatment of Patients with Pulmonary Tuberculosis

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By oxidation-reduction assay, the viability of *Mycobacterium tuberculosis* in sputum specimens was evaluated. The technique is based on the Alamar Blue and Malachite Green dyes, which change their color in response to *M. tuberculosis* growth. The method is simple, permits visual reading of results, and is applicable for laboratories with limited resources.

Usually, 30% of patients receiving antituberculosis therapy will have their sputum cultures converted to negative before the smear conversion (2, 7, 11, 13, 15). The phenomenon is due to the presence of nonviable mycobacteria or atypical mycobacterial colonization (1, 16, 20). These cases have to be differentiated from treatment failure patients who have persistently positive smear and culture results after completion of therapy. Recent advances in technology introduced many rapid and reliable methods to differentiate between live and dead acid-fast bacilli in clinical specimens (9, 17, 19). However, due to their high cost and equipment requirements, the new methods are simply not feasible in most laboratories of developing countries (4, 5, 6). These countries use traditional solid-culture-based methods, such as Lowenstein-Jensen (LJ) and Ogawa (14), which are very time consuming. Consequently, the tuberculosis (TB) physician makes the decision based on the microscopy results. Therefore, supplementary rapid and reliable methods are desirable for TB laboratories with limited resources. In 1995, D. Yajko et al. (21) used an oxidation-reduction indicator, Alamar Blue, which changes its color in response to the growing organisms. He used a microplate Alamar Blue assay for evaluation of antimicrobial susceptibility of *Mycobacterium tuberculosi*s. The Alamar Blue in microtiter wells was initially blue in an oxidizing state and became pink in the reduced form (3, 12, 18). In the present study, a tube version of the Alamar Blue assay was used to evaluate the viability of *M. tuberculosi*s in serial sputum specimens from persistently smear-positive pulmonary TB patients receiving antituberculosis treatment. In another similar assay, we used another oxidation-reduction dye, Malachite Green, for the first time. Malachite Green is a triphenylmethane dye and has a dark green color but becomes colorless during *M. tuberculosi*s metabolism. The accuracy and feasibility of the Alamar Blue and Malachite Green assay were compared with those of standard LJ culture medium. In total, 110 newly diagnosed TB patients were enrolled in this study. From each patient, seven sputum specimens were collected on day zero and then monthly for 6 months. During the study period, 35 patients (31.8%) were excluded because of negative smear. Another 15 patients (13.6%) were excluded because of their smear converting from positive to negative by the third month of treatment, and an additional 20 patients (18%) were excluded because of other criteria (they were not able to produce enough sputum or there was no bacteriological follow-up). Consequently, the number of understudy cases decreased to 40 (36.3%) who had positive smear results throughout the treatment courses. For negative controls, the sputum specimens were collected from 40 patients diagnosed with diseases other than tuberculosis (including allergy, asthma, and lung cancer). All specimens were digested and decontaminated of other bacteria by the standard *N*-acetyl-L-cysteine—NaOH method (10), and the remaining sediments were resuspended in 2 ml of phosphate-buffered saline (0.01 M; pH 7.2). Two hundred fifty microliters of this suspension was inoculated into an LJ culture slant, and the remaining was mixed with 2 ml of 7H9GC broth (4.7 g of Middle-brook 7H9 broth base, 20 ml of 10% glycerol, 1 g of Bacto Casitone, 880 ml of distilled water, and 100 ml each of oleic acid albumin, dextrose, and catalase, all from Difco Laboratories, Detroit, Mich.). Then, equal amounts of mixture were transferred into two 15-ml falcon tubes (Nalge; Nunc International, Naperville, Ill.). In the first tube, the oxidation-reduction assay was started by adding 250 μl of a freshly prepared 1:1 mixture of 10× Alamar Blue (Accumed International, Westlake, Ohio) reagent and 10% Tween 80 (8). In the second tube, the reaction was started by adding 250 μl of Malachite Green (Merck) with a concentration of 0.05 mg/ml. The tubes were sealed with parafilm and incubated at 37°C. The color of the tubes was recorded daily for 8 weeks. In tubes containing Alamar Blue indicator, blue color was interpreted as no growth and pink color was interpreted as growth. In Malachite Green tubes, dark green color was interpreted as no growth and light green or colorlessness scored as growth.

The mean time and positivity difference of Alamar Blue, Malachite Green, and LJ culture media were assessed using the *t* test and paired *t* test. A P value of <0.05 was regarded as significant.

Overall, out of 280 positive smear samples, the LJ culture

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TABLE 1. Results of culture for persistently smear-positive specimens and a control group

Culture medium[a]	No. (%) of correctly identified specimens[b]		
	S+/C+[c]	S+/C−[c]	S−/C−[d]
LJ culture slants	124 (44.2)	151 (53.9)	37 (92)
Alamar Blue culture tubes	118 (42)	141 (50)	33 (82)
Malachite Green culture tubes	116 (41)	140 (50)	35 (87)

[a] The sensitivities of the culture media were as follows: for LJ culture slants, 99%; for Alamar Blue culture tubes, 95%; and for Malachite Green culture tubes, 93%.

[b] S+/C+, smear and culture positive; S+/C−, smear positive and culture negative; S−/C−, smear and culture negative.

[c] A total of 280 patient sputum specimens were tested.

[d] A total of 40 control sputum specimens were tested.

was positive in 124 (44%) cases and negative in 151 cases (53%). In comparison to LJ culture medium, the recovery rates in smear positivity/culture positivity with Alamar Blue and Malachite Green culture media were 118 and 116, out of 124, respectively. The results also demonstrate (Table 1) that the number of correctly identified smear-positive/culture-negative sputa with oxidation-reduction assay was almost similar to that with the LJ culture medium; out of 151 smear-positive/culture/negative cases, 141 and 140 were correctly detected by the Alamar Blue and Malachite Green culture tubes. Similarly, in the control group (smear negative/culture negative), the accordance between Alamar Blue and LJ culture media was 33 out of 37 (82.5%) and in Malachite Green was 35 out of 37 (87.5%). The overall contamination rate in both groups (patients and controls) was 2.5% by LJ, 4.6% by Malachite Green, and 5.3% by Alamar Blue. The sensitivity of LJ was 99%, that of the Alamar Blue assay was 95%, and that with Malachite Green was 93%. The specificities were 100, 93, and 92%, respectively. The mean time required to get a positive signal by Alamar Blue culture medium was 9 days, and that by Malachite Green culture medium was 11 days. In the standard LJ culture medium, the average detection time was 27 days ($P < 0.05$). The experiments also showed that there is a direct relation between the number of bacilli in the processed specimens and the time needed for detection of positive signals in oxidation-reduction culture media. For example, in specimens with microscopy results of 3 positive (i.e., the number of acid-fast bacilli per millimeter of sputum was approximately 500,000 or more), the required time for cultures to show a positive signal was between 5 and 9 days, whereas it was between 15 and 19 days for specimens with scanty microscopy results ($P < 0.05$).

The results showed that both oxidation-reduction assays have the potential of becoming the routine method for assessing chemotherapeutic efficacy for TB patients, although the Malachite Green culture medium is more cost effective and readily available than the Alamar Blue culture medium. In conclusion, the oxidation-reduction assay will allow rapid identification of dead and live acid-fast bacilli in the sputum specimens, which may decrease the length of the hospital stay and eliminate the need for expensive diagnostic procedures, especially in TB laboratories with limited resources.

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