JOURNAL OF CLINICAL MICROBIOLOGY, May 1990, p. 1068–1070
0095-1137/90/051068-03$02.00/0

Vol. 28, No. 5

Modified Method for Testing the Quality of Albumin-Containing Enrichments Used in Growth Media for Mycobacteria

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Received 13 September 1989/Accepted 19 January 1990

Many commercially available media for cultivation of mycobacteria have failed to support the growth of these organisms. This is especially true of media prepared with albumin-containing enrichments. Earlier, we developed a method for rapid identification of good albumin enrichments for agar-based media used to test the susceptibility of tubercle bacilli to pyrazinamide. The method was modified to make testing of the acceptability of albumin enrichments for primary isolation media for mycobacteria possible. We describe here a simple turbidimetric test using a specific *Bacillus subtilis* strain to assay quickly (24 h) different lots of albumin-containing enrichments that may be used in the preparation of growth media for mycobacteria.

Commercially available albumin-containing enrichment supplements of poor quality for use in Middlebrook agar-based media continue to be a concern for mycobacteriologists. In a 15-year study, Guthertz et al. revealed seven instances (30%) of poor performance among 23 albumin supplements tested (2). Earlier, we described a rapid turbidimetric test using *Bacillus subtilis* to determine the suitability of different lots of albumin-dextrose-catalase (ADC) enrichment for use in drug susceptibility testing with 7H10 agar medium at pH 5.5 (1). For 5 years, we monitored our ADC by this test and demonstrated that the turbidimetric test results were a reliable indicator of the growth we could expect on the agar at low pH. For the last 7 years, we have used a slightly modified procedure to evaluate growth-supporting quality and to control the purchase of albumin-containing enrichment supplements, i.e., ADC and oleic acid-albumin-dextrose-catalase (OADC) used in preparing the 7H series of mycobacterial liquid or agar media. Few laboratories use any quality control procedure before purchasing the albumin-containing enrichments. One of us (G.P.K.) has received numerous complaints from clinical laboratory personnel about the inability of many agar-based media to support growth of mycobacteria. Guthertz et al. (2) had shown that bad lots of either agar base or enrichment could contribute to poor growth of mycobacteria on 7H10 media. Our modified *B. subtilis* test permits a rapid identification of one of the medium components, the albumin-containing enrichment. We report here a simple turbidimetric procedure to pretest lots of these enrichments so that they may be used to prepare primary isolation media.

Test organism. Grow *B. subtilis* ATCC 23059 in heart infusion broth (HIB) for 24 h at 37°C. Use this broth to inoculate stock slants of brain heart infusion agar. Grow stock slants for 4 to 7 days at 35°C, and then store them (for up to 1 year) at normal refrigerator temperature (4°C) until needed.

A standardized test suspension of *B. subtilis* can be prepared from the refrigerated stock slant. Inoculate a loopful of culture material into a tube (16 by 125 mm) containing 5 ml of HIB, and incubate the culture material for 24 h at 35°C. Use sterile distilled water to adjust the optical density (OD) to 0.3 at a wavelength of 650 nm.

Test performance. (i) Controls. (a) Prepare a spectrophotometer zero set tube by mixing 4.1 ml of sterile distilled water and 0.5 ml of sterile HIB. This tube is used to adjust the spectrophotometer to zero.

(b) Prepare a *B. subtilis* background tube by mixing 4 ml of sterile distilled water, 0.5 ml of sterile HIB, and 0.1 ml of standardized suspension of *B. subtilis*. This tube is used to determine the growth of *B. subtilis* in HIB alone.

(c) Prepare a *B. subtilis* negative control by mixing 0.1 ml of *B. subtilis* standardized suspension, 0.5 ml of sterile HIB, and 4 ml of previously tested sterile enrichment that did not pass the test described here.

(d) Prepare a *B. subtilis* positive control by mixing 0.1 ml of *B. subtilis* standardized suspension, 0.5 ml of sterile HIB, and 4 ml of previously tested sterile enrichment that passed the test described here. Both the negative and positive control enrichments may not be immediately available. Negative and positive controls may be selected from enrichments that have failed or passed other quality control assays, such as those proposed by Guthertz et al. (2). Although the OD variations for good and bad enrichments are theoretically unlimited, in our experience, negative control enrichments commonly yield final OD increases of ≤ 0.08, while positive controls provide OD increases of ≥ 0.16. Once a supply of each kind is found, a large volume should be set aside (and replenished when depleted) for future control use.

(e) Prepare sterile controls for each enrichment to be tested by mixing 4 ml of the enrichment, 0.5 ml of sterile HIB, and 0.1 ml of sterile distilled water. This control acts as a sterility indicator for the enrichment, the HIB, and the water used during the test.

(ii) Set up sterile enrichments for the assay of growth-supporting ability as described in section (i) (e) above, but substitute 0.1 ml of the *B. subtilis* standardized suspension for the 0.1 ml of sterile distilled water.

(iii) Assemble all controls and test sets as described above, tightly cap the tubes, and mix the suspension thoroughly. Set the spectrophotometer to zero with the zero set tube de-

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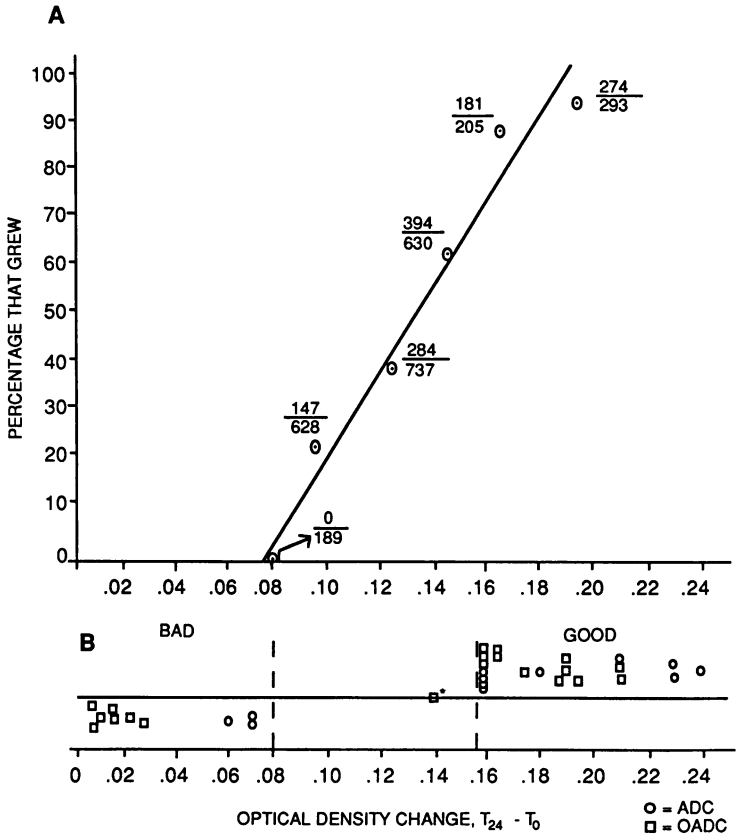

FIG. 1. Correlation of OD change ($T_{24} - T_0$) of *B. subtilis* culture with abilities of albumin-enriched media to support growth of mycobacteria. (A) Regression line of percentage of *M. tuberculosis* strains growing on pH 5.5 medium made with enrichments exhibiting different OD increases; fractions by each point (⊙) indicate the number growing over the number tested. Total number of strains, 2,682. (B) Distribution of OD increases of good and bad batches of ADC (○) and OADC (□) enrichments. See text for details. Medium made with the lot marked * (OD increase, 0.14) supported the growth of the quality control strains of *M. tuberculosis* but greatly inhibited the growth of both *M. gordonae* and *M. kansasii*.

scribed in section (i) (a) above. Record baseline OD readings for all tubes in the spectrophotometer set at 650 nm (T_0 reading).

(iv) Incubate all tubes at 35°C for 24 h.

(v) After 24 h, read and record the spectrophotometric OD readings on all tubes (T_{24} reading).

(vi) Determine the difference in OD by subtracting the baseline T_0 reading from the T_{24} reading.

(vii) Determine the $T_{24} - T_0$ reading of the background *B. subtilis* tube, and then subtract this reading from the $T_{24} - T_0$ reading of each enrichment tested; this represents a true OD reading.

(viii) Interpret the test as follows.

(a) An enrichment under assay that exhibits a final $T_{24} - T_0$ OD reading of 0.16 or greater [i.e., the $T_{24} - T_0$ reading of the *B. subtilis* background tube, described in section (i) (b) above, has been subtracted from the $T_{24} - T_0$ reading of the assayed enrichment] is considered suitable for use.

(b) Enrichments that yield true OD increases of less than 0.16 are not suitable for primary isolation growth media.

(c) If an increase of more than 0.009 OD unit occurs in the sterile controls, contamination should be suspected and the test should be repeated.

Initially, studies were conducted with different strains and species of the genus *Mycobacterium* (*Mycobacterium tuberculosis* H37Rv [TMC 102; Trudeau Mycobacterial Culture Collection] and H37Ra [provided by Kuni Takayama, Veterans Administration Hospital, Madison, Wis.] and *Mycobacterium smegmatis* ATCC 607 [American Type Culture Collection, Rockville, Md.]). The test using *B. subtilis* W-23 (originally from Gary Best, Medical College of Georgia, Augusta; the comparable strain ATCC 23059 works equally well, although final ODs may be lower) was safer and faster and always yielded results comparable to those obtained with the test strains of *M. tuberculosis*.

By comparing OD readings with the percentage of *M. tuberculosis* strains that grew at pH 5.5, we determined that a $T_{24} - T_0$ OD increase of 0.16 supported the growth of 75% or more of all *M. tuberculosis* strains tested at this low pH (1). Even with the best ADC enrichment tested, we found that 6 to 10% of *M. tuberculosis* strains failed to grow on media at this low pH when such media were used to test their susceptibility to pyrazinamide. In similar studies, other investigators have reported that 4 to 28% of strains failed to grow (3–5).

Our experience has shown that when cultures of *M. tuberculosis* are grown on 7H10 agar made with good enrichment, the percentage of strains that grow on media adjusted to pH 6.8 is much higher than that on media at pH 5.5 and approaches 99%. The inability of some strains to grow at pH 5.5 seems to be related to their multiply drug-resistant nature, although this has been questioned by Tarrand et al. (4). On the basis of an OD increase of 0.16 established for pH 5.5 media, we have adopted the same cutoff value for media

having a more neutral pH. Evidence to support this same cutoff value has been strengthened by our agreement to test some enrichments that others have found, in actual use, to be either good or bad.

The turbidimetric data for medium enrichments tested at both pH 5.5 and pH 6.8 are summarized in Fig. 1. The line graph is a linear regression curve for 2,682 strains tested on different batches of 7H10 agar (pH 5.5) prepared with seven different ADC enrichments. Also in Fig. 1 are two regions labeled "good" and "bad." These represent batches of OADC and ADC that were evaluated for use either with 7H10 agar plates (pH 6.8) for primary isolation or drug testing or with 7H10 agar (pH 5.5) for pyrazinamide susceptibility tests. With one exception, all of the good batches yielded an OD increase of >0.16 for the *B. subtilis* culture. The truly bad batches yielded OD increases of 0.08 or less. The one batch of OADC that had an OD increase of 0.14 supported growth of the two test strains of *M. tuberculosis* but proved greatly inhibitory for the single test strains of *Mycobacterium gordonae* and *Mycobacterium kansasii*. Medium made from this enrichment may still be suitable for work with subcultures but may be questionable for use in primary isolation.

For 7 years, we have used the *B. subtilis* assay at the Centers for Disease Control to test enrichment supplements before accepting them for preparing media. During this time we tested 32 lots of different enrichments (12 ADC and 20 OADC). We rejected 25% (3 of 12) of the ADC enrichments and 35% (7 of 20) of the OADC enrichments (Fig. 1). Using a more stringent quality control test that also measures binding of antituberculosis drugs, Guthertz et al. (2) reported a 30% rejection rate for the OADC component of their drug susceptibility test media.

An example of the kind of inhibition encountered in 7H10 agar (pH 6.8) is shown in Fig. 2. The set of seven plates at the top of the figure was prepared from a good batch of OADC and inoculated with serial 10-fold dilutions of a wild-type isolate of *M. gordonae* (ranging from undiluted to a 10^{-6} dilution). Duplicate inocula on the 10^{-6} plate provided an average of 23 colonies from a 0.1-ml inoculum (i.e., 2.3×10^8 colonies per ml in the undiluted inoculum). In contrast, when the same inocula were placed on the seven plates at the bottom of the figure (made from bad OADC), only two colonies grew on the 10^{-1} dilution, a $>10^6$-fold reduction in countable colonies. Both of these OADC enrichments were being sold to the general scientific community.

Since we supplied this system to several other investigators, we have been informed that some are able to achieve $T_{24} - T_0$ OD readings much higher than the near maximum of 0.24 to 0.26 that we have observed and reported here (Fig. 1). This may be due, at least in part, to the degree of smoothness of the test suspension of *B. subtilis* or to the strain; until recently, we were not specific in our source of this culture, but we have since recommended the American Type Culture Collection *B. subtilis* strain listed in this publication as one that generally yields a uniformly homogeneous suspension when fully grown. Because of this possible difficulty, we suggest that each investigator establish his or her own operating definitions of good and bad.

At this time, we do not know which component of a particular enrichment is responsible for the good (or bad) qualities of the enrichment. Whatever the component, its presence (or absence) in a specific lot of enrichment correlates well with the ability of that enrichment to support (or inhibit) growth of both mycobacteria and the test strain of *B. subtilis*.

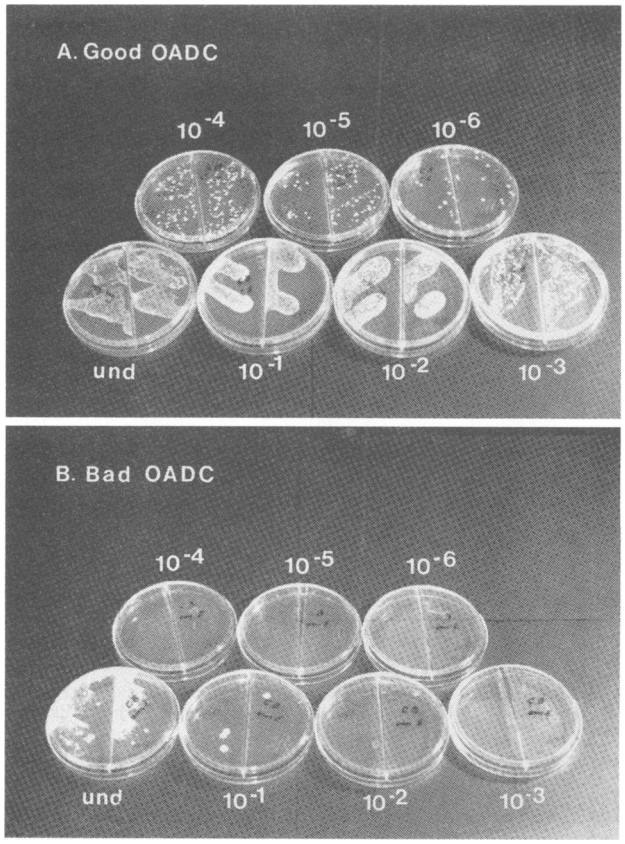

FIG. 2. Growth of identical inocula of *M. gordonae* on plates of 7H10 agar made from good and bad batches of OADC enrichment. See text for details. und, Undiluted.

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