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# Culture of specimens other than sputum for Mycobacteria

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SYNOPSIS A comparison has been made of Lowenstein-Jensen medium, with or without added pyruvate, 7H11 oleic acid-albumin agar slopes made selective by the addition of antibiotics, and selective Kirchner medium in the isolation of tubercle bacilli from specimens other than sputum. A combination of a standard mild preliminary treatment with sulphuric acid and selective medium reduced the contamination rate to 0·1%. Neither acid treatment nor antibiotics appeared to reduce the isolation rate. An effective procedure is to treat all specimens (except cerebrospinal fluid) with acid and culture on two Lowenstein-Jensen slopes (one with pyruvate), a 7H11 selective slope, and in two bottles of selective Kirchner medium.

Marks (1972) has recommended that specimens other than sputum should be cultured for mycobacteria after treatment with sulphuric acid, graded in concentration and exposure period according to the degree of contamination. The degree of contamination was assessed by preliminary examination of a Gram-stained film of the material or of a wet preparation of urine deposits—procedures that are often inconvenient. Mitchison, Allen, and Lambert (1973) described the use of medium made selective by the addition of antibiotics without prior treatment of the specimen with acid or alkali. They obtained fairly satisfactory results with tissues, which are seldom heavily contaminated. We report here an investigation of the combined use of treatment with acid for a standard short period and selective medium in the culture of all types of specimen other than sputum. The study included all of the 2540 specimens received during the 18-month period starting in June 1972.

## Materials and Methods

### CULTURE MEDIA

The media used were Lowenstein-Jensen slopes without potato starch (LJ) (Cruickshank, 1965), LJ slopes containing 0·5% sodium pyruvate (LJ + P), 7H11 oleic acid-albumin agar with malachite green (Bacto, 0838), Kirchner medium with phenol red but without penicillin (OK medium) (Oxoid, CM193), and Kirchner medium modified by the addition of 0·5 g/l casein hydrolysate (Oxoid tryptone, L42) and the substitution of 10% calf serum for horse serum (NK medium). They were made selective by the addition of polymyxin B 200 units/ml, carbenicillin 100 µg/ml, trimethoprim 10 µg/ml and amphotericin B 10 µg/ml (Mitchison, Allen, Carrol, Dickinson, and Aber, 1972). All media were dispensed in screw-capped (universal) bottles, the slopes in 5 ml and the liquid media in 10 ml amounts.

### TREATMENT OF SPECIMENS

Centrifugation was for 15 min at 3000 rpm. Specimens were prepared for allocation to treatment with or without acid as follows. Tissues were ground in polytetrafluorethylene grinders or chopped with scissors and then macerated in a Griffith's grinder to make 2 ml suspensions. Aspirates were centrifuged and the deposits resuspended in 1-2 ml sterile water. Both types of suspension were then divided into two portions. Two samples of urine, each of about 20 ml, were centrifuged and the deposit resuspended in 0·5-1 ml sterile water. Using lists of random numbers, one of the pair of suspensions or urine samples was then treated with an equal volume of 5% (v/v) $H_2SO_4$ for 15 minutes. About 15 ml sterile water (5 ml for cerebrospinal fluids) was added, the tube was centrifuged and sterile water was added to the deposit to make up the volume to the same as that of the sample not treated with acid. The following media were

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inoculated from the acid-treated suspension: one slope each of LJ, LJ + P, and selective 7H11 medium (S7H11) inoculated with 2 loopfuls; two bottles of NK medium each inoculated with 0·1 ml, one selective (SNKO.1) and one without antibiotics (NKO.1); one bottle of selective NK medium inoculated with 0·4-0·8 ml, the remainder of the suspension (SNKO.8). The sample not treated with acid was inoculated into the following media: S7H11, SNKO.1, selective OK medium (SOKO.1), and SNKO.8. Pus swabs were inoculated onto a S7H11 slope and, after immersing in 5% (v/v) $H_2SO_4$ for 15 min and sterile water for 5 sec, onto LJ, LJ + P, and S7H11 slopes; the tip was then broken off and dropped into SNK medium. The order of inoculation of the slopes or bottles within each group of media inoculated with a loop, with 0·1 ml suspension, or with the remainder of the suspension, was randomized, except for pus swabs.

All media were incubated at 37°C without added $CO_2$ and were examined weekly for 7 to 8 weeks. Each Kirchner bottle was subcultured onto an LJ slope following the reading at 5 to 6 weeks, if not positive earlier. Cultures of mycobacteria were identified by standard methods, including a test for niacin production.

## Results

The results according to the type of specimen examined are set out in table I. The contamination rates on media inoculated with urine which had not been treated with acid were high in the initial part of the study (phase I), so that subsequent urines were only treated with acid (phase II). Positive cultures of *Mycobacterium tuberculosis* were obtained from 60 (2·4%) of the 2540 specimens, most frequently from pus, tissues, and cerebrospinal fluid (from a single patient) and least frequently from endometrial biopsies. Only three specimens were contaminated in all media.

The number of positive isolates of *M. tuberculosis* and the contamination rates for the separate media are set out in table II. Similar numbers of positive cultures were obtained on ACID/LJ and ACID/S7H11 slopes and the mean incubation periods for the 37 specimens positive on both slopes were also similar, being 3·1 and 3·2 weeks, respectively. Positive cultures were obtained more often from Kirchner medium than from slopes, especially when the Kirchner medium was seeded with a large inoculum. For example, out of 2472 specimens other than pus swabs, a positive culture was obtained from 36 ACID/S7H11 slopes, from 41 ACID/SNKO.1 bottles, and from 43 ACID/SNKO.8 bottles. Treatment with acid was less effective than selective medium in reducing contamination. Thus contamination occurred in 24·7% of 227 phase I urines in ACID/NKO.1 bottles and in 16·7% in SNKO.1 bottles, the proportions for specimens other than urine or pus swabs being 5·4% and 1·6%, respectively. When acid treatment and selective medium were combined, the contamination rates were very low, being only 0·7% for ACID/S7H11 slopes, 0·3% for ACID/SNKO.1 bottles, and 0·8% for ACID/SNKO.8 bottles out of the 2540 specimens. The original and modified versions of the Kirchner medium yielded similar isolation and contamination rates.

Table III sets out the results of all possible comparisons of the isolation rates between pairs of media after exclusion, for each comparison, of specimens which were contaminated in either medium and of the results from pus swabs, for which the order of inoculation of the media was not randomized. That the acid treatment had little

| *Type of Specimen* | *Total Specimens* | *Positive (Any Medium)* | *No. of Media per Specimen* | *Media Contaminated* | | |
|---|---|---|---|---|---|---|
| | | | | *10-25%* | *25-90%* | *100%* |
| | | | | *No. of Specimens* | *No. of Specimens* | *No. of Specimens* |
| Urine: phase I | 227 | 3[1] (1·3%) | 10 | 83 (36·6%) | 52 (22·9%) | 0 (0%) |
| Urine: phase II | 1219 | 12[1] (1·0%) | 6 | 112 (9·2%) | 70 (5·7%) | 1 (0·1%) |
| Pus swabs | 68 | 7 (10·3%) | 5 | 5 (7·4%) | 1 (1·5%) | 0 (0%) |
| Endometrial biopsy | 419 | 1 (0·2%) | 10 | 19 (4·5%) | 2 (0·5%) | 0 (0%) |
| Chest fluids | 249 | 9 (3·6%) | 10 | 16 (6·4%) | 14 (5·6%) | 1 (0·4%) |
| Cerebrospinal fluid | 54 | 6 (11·1%) | 10 | 2 (3·7%) | 0 (0%) | 0 (0%) |
| Other fluids | 135 | 2 (1·5%) | 10 | 6 (4·4%) | 2 (1·5%) | 0 (0%) |
| Lymph glands | 58 | 5 (8·6%) | 10 | 3 (5·2%) | 0 (0%) | 0 (0%) |
| Tissues and pus | 111 | 15 (13·5%) | 10 | 15 (13·5%) | 8 (7·2%) | 1 (0·9%) |
| Total | 2540 | 60 (2·4%) | | 261 (10·3%) | 149 (5·9%) | 3 (0·1%) |

Table I *Isolation of* M. tuberculosis *and contamination rate according to type of specimen*

[1] In addition five specimens yielded cultures of mycobacteria other than tubercle bacilli, three in phase I and two in phase II.

| *Treatment and Medium* | *Urine I* | | *Urine II* | | *Pus Swabs* | | *Other Specimens* | |
|---|---|---|---|---|---|---|---|---|
| | *Positive*[1] | *Contaminated*[2] | *Positive* | *Contaminated* | *Positive* | *Contaminated* | *Positive* | *Contaminated* |
| ACID/LJ | 3 | 9 | 11 | 46 | 6 | 3 | 22 | 20 |
| ACID/LJ + P | 3 | 19 | 11 | 55 | 6 | 4 | 21 | 22 |
| ACID/S7H11 | 3 | 0 | 11 | 8 | 4 | 0 | 22 | 10 |
| S7H11 | 0 | 49 | — | | 3 | 0 | 22 | 13 |
| ACID/NKO.1 | 1 | 56 | 11 | 129 | — | | 23 | 55 |
| ACID/SNKO.1 | 3 | 0 | 12 | 4 | 5 | 0 | 26 | 4 |
| SNKO.1 | 2 | 38 | — | | — | | 29 | 16 |
| SOKO.1 | 2 | 36 | — | | — | | 28 | 12 |
| ACID/SNKO.8 | 2 | 4 | 11 | 10 | — | | 30 | 7 |
| SNKO.8 | 2 | 61 | — | | — | | 33 | 17 |
| Total Specimens | 227 | | 1219 | | 68 | | 1026 | |

Table II *Isolation of* M. tuberculosis *and contamination rate according to method of treatment of specimen and culture medium*

[1]Number of specimens positive on medium specified.
[2]Number of specimens contaminated on medium specified.

| *Negative on Column Medium Positive on Row Medium* | *Positive on Column Medium, Negative on Row Medium* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *ACID LJ* | *ACID LJ + P* | *ACID S7H11* | *S7H11* | *ACID NKO.1* | *ACID SNKO.1* | *SNKO.1* | *SOKO.1* | *ACID SNKO.8* | *SNKO.8* |
| ACID/LJ | – | 2 | 3 | 2 | 4 | 6 | 8 | 8 | 9[2] | 12[2] |
| ACID/LJ + P | 3 | – | 4 | 4 | 4[2] | 6[2] | 9[2] | 9 | 9[2] | 12[2] |
| ACID/S7H11 | 3 | 3 | – | 2 | 4 | 7 | 8 | 9 | 9[2] | 12[2] |
| S7H11 | 2 | 3 | 3 | – | 5 | 5 | 7[2] | 7 | 8[2] | 11[2] |
| ACID/NKO.1 | 1 | 0[2] | 1 | 2 | – | 2 | 5 | 6 | 6 | 9[2] |
| ACID/SNKO.1 | 1 | 0[2] | 2 | 1 | 1 | – | 4 | 6 | 6 | 8[2] |
| SNKO.1 | 2 | 2[2] | 2 | 0[2] | 2 | 2 | – | 4 | 6 | 7 |
| SOKO.1 | 3 | 3 | 4 | 2 | 3 | 5 | 5 | – | 4 | 8 |
| ACID/SNKO.8 | 1[2] | 0[2] | 1[2] | 1[2] | 1 | 3 | 4 | 1 | – | 5 |
| SNKO.8 | 1[2] | 0[2] | 1[2] | 0[2] | 1[2] | 1[2] | 2 | 2 | 2 | – |

Table III *Matrix of numbers of specimens positive on one medium and negative on the other*[1]

[1]Specimens with either medium contaminated are excluded from each comparison.
[2]Contrast attains statistical significance ($P < 0.05$).

bactericidal activity against *M. tuberculosis* is shown by comparing the results on selective medium with and without acid. Thus three specimens were positive on ACID/S7H11 and negative on S7H11 while two specimens were positive on S7H11 and negative on ACID/S7H11. The corresponding numbers for the SNKO.1 media were two positive on ACID/SNKO.1 only and four on SNKO.1 only, and for SNKO.8 media, two on ACID/SNKO.8 only and five on SNKO.8 only. Comparison of ACID/NKO.1 with ACID/SNKO.1 also suggests that the antibiotics in the selective media did not inhibit the growth of *M. tuberculosis*. Positive results were obtained more often from Kirchner media inoculated with the large remainder volume (ACID/SNKO.8 and SNKO.8) than from the four slopes inoculated with a loop, the contrast achieving statistical significance in all eight comparisons. Isolates were also obtained more frequently from the Kirchner media inoculated with 0·1 ml suspension than from the slopes, the contrast being significant in four of the 16 comparisons. Finally, Kirchner medium inoculated with the large remainder volume yielded more positives than the same medium inoculated with 0·1 ml, though the contrast was significant in only two of the eight comparisons.

The results that would have been obtained had various combinations of media been employed are shown in table IV. If specimens had been treated with acid and then inoculated only onto LJ and LJ + P slopes, 45 specimens would have yielded positive culture and 51 (2·0%) would have been contaminated. Inclusion of an S7H11 slope would have substantially reduced the contamination rate to 0·3%, and would have slightly increased the yield of positive cultures. Inclusion of the two SNKO.1 and SNKO.8 bottles would have increased the yield of positive cultures to 55, but the use of both the S7H11 slope and the SNK bottles would have had only a minor further advantage. The inclusion of

| Media Used[1] | Urines and Pus Swabs | | Other Specimens | | All Specimens | |
|---|---|---|---|---|---|---|
| | Positive[2] | Contaminated[3] | Positive | Contaminated | Positive | Contaminated |
| LJ, LJ + P | 21 | 37 | 24 | 14 | 45 | 51 |
| LJ, LJ + P, S7H11 | 21 | 2 | 26 | 5 | 47 | 7 |
| LJ, LJ + P, SNK[4] | 22 | 3 | 33 | 2 | 55 | 5 |
| LJ, LJ + P, S7H11, SNK[4] | 22 | 1 | 34 | 2 | 56 | 3 |
| LJ, LJ + P, S7H11, SNK[4], SNK[4] (no acid) | — | — | 38 | 2 | — | — |
| Total specimens | 1514 | | 1026 | | 2540 | |

Table IV *Isolation of* M. tuberculosis *and contamination using various selections of media*

[1]Specimens treated with acid, except where indicated.
[2]Number of specimens positive on any medium.
[3]Number of specimens contaminated on all media.
[4]SNKO.1 and SNKO.8

two more SNK bottles inoculated without previous acid treatment from specimens other than urines or pus swabs would have yielded an additional four positive results. Thus although better results followed from an increase in the number of media inoculated from each specimen, the biggest advantage was obtained from inclusion of the SNK bottles in addition to slopes.

## Discussion

Several considerations influence the choice of the best procedure for the routine culture of specimens other than sputum for mycobacteria. First, there are practical advantages in a standardized procedure which does not require a special assessment, such as examination of a Gram-stained film, to determine the subsequent treatment of the specimen. Secondly, tissue specimens and aspirates are usually obtained at operation and cannot often be repeated. The laboratory should therefore use efficient culture methods yielding the lowest possible contamination rates. These two considerations suggest that specimens should be given an initial mild treatment with acid and then be cultured on more media than are used for sputum, including several made selective by the addition of antibiotics. The combination of acid treatment and selective medium resulted in remarkably low contamination rates in the present study and neither acid treatment nor the antibiotics appeared to reduce the number of positive isolations. Thirdly, the results indicate that more isolations would be obtained by the inclusion of Kirchner medium in addition to slopes. Kirchner medium with penicillin was found superior to Lowenstein-Jensen medium in an early cooperative study (Public Health Laboratory Service, 1958). Mitchison, *et al* (1973) found selective Kirchner medium inferior to selective 7H11 slopes, but the study was confined to the culture of tissues and the poor results with Kirchner medium may have been due to the inhibitory action of large inocula of tissue.

A satisfactory routine procedure for all specimens other than sputum would be to treat with acid as described, using a single concentration and exposure period, except that acid treatment seems unnecessary for cerebrospinal fluid. The material would then be inoculated onto two slopes of Lowenstein-Jensen medium (one containing pyruvate), a selective 7H11 slope, and into two bottles of Kirchner medium. Slopes are of value because they yield identifiable positive cultures earlier than liquid medium. The use of one Lowenstein-Jensen slope containing pyruvate is a common practice in sputum culture, since pyruvate increases the size of colonies of *M. bovis* and the isolation rate of *M. tuberculosis*, but may inhibit *M. kansasii* (Stonebrink, 1958; Marks, 1963; Hughes, 1966; Cavanagh and Keyes, 1968). Inclusion of a selective 7H11 slope is suggested because of the low contamination rate with this medium, though positive results were not obtained earlier than on Lowenstein-Jensen slopes as has been claimed for the closely similar 7H10 medium by Lorian (1967) and Liu, McGregor, Faucher, Jinks, Miller, Green, and Liu (1973). The use of this procedure would have yielded 56 of the 60 positive specimens in the study with a contamination rate of 0·1%. Had two further bottles of Kirchner medium been inoculated without previous acid treatment, from tissues and aspirates only, the full yield of 60 positive specimens would have been obtained.

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