Laboratory Microscopy 2

Computer Files and Analyses of Laboratory Data from Tuberculosis Patients. II. Analyses of Six Years' Data on Sputum Specimens[1-4]

Lab dx - microscopy misc

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SUMMARY

Laboratory data on sputum specimens from patients with pulmonary mycobacterioses between 1968 and 1973 were analyzed. Specimens were cultured on Middlebrook 7H10 and/or 7H11 medium; blue light fluorescence microscopy was used to examine specimen smears. An admission series of 6 sputum specimens detected 94.7 per cent of all culture-positive patients. Only 62 per cent of patients who were culture positive in the admission series would have been detected by smear alone. Quantitative agreement of smear and culture results was seen only in the smear-*numerous* (>2 bacilli per high-power field), culture-*numerous* (>100 colonies per plate) category. Qualitative agreement of smear and culture was 71 per cent.

A total of 123 smear-positive and culture-negative specimens, representing 9 per cent of the total number of positive smears, was found among 6,251 sputum specimens from 270 culture-positive patients. At least one smear-positive and culture-negative specimen was obtained from 23 per cent of the patients evaluated. During this period, improved housekeeping measures and drying of culture plates reduced the culture contamination rate from 9.8 to less than 5 per cent.

Introduction

A computer management system for handling mycobacteriologic data on specimens from tuberculous patients has been described (1). Ready access to a 6-year, in-depth mycobacteriologic data file on inpatients with tuberculosis at Fitzsimons Army Medical Center (FAMC) made possible a re-evaluation of the diagnostic methodology at this laboratory.

This report is based on data from homogenized and concentrated sputum specimens and pertinent file information of laboratory operations, i.e., optimal number of sputum specimens needed to detect positivity, optimal culture incubation times, smear-culture correlation, and culture contamination rates.

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Materials and Methods

Specimen collection. A series of 6 early morning sputum specimens was collected on consecutive days from patients admitted to the Tuberculosis Service; thereafter, specimens were submitted at regular intervals until the patient was discharged. Specimens were processed within 3 to 4 hours after collection or, rarely, were held overnight in the refrigerator and processed the following day.

Decontamination. A standard *N*-acetyl-L-cysteine method (2) using final sodium hydroxide concentrations of 3 to 2 per cent was used. The buffer-neutralized sediment was resuspended in 3 to 4 ml of

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0.2 per cent albumin-M/15 phosphate buffer, pH 6.6, for inoculation.

Smears. A smear, approximately 1.5 × 3 cm, was prepared from 1 drop of concentrated sediment. Smears were air dried, exposed to 10 per cent formalin fumes for 30 min (3), and heated on a slide warmer at 65° C for 30 min.

Smears were stained for 15 min with 0.1 per cent auramine O (0.1 g of auramine O in 10 ml of 95 per cent ethanol, diluted to 100 ml with 3 per cent [vol/vol] aqueous phenol solution) (4), rinsed with tap water, decolorized for 2 min with 0.5 per cent hydrochloric acid-ethanol, rinsed, quenched for 3 min with 0.5 per cent potassium permanganate, rinsed, and air dried.

Microscopy. Blue light visualization of acid-fast bacilli (AFB) was performed as previously described (5) using a Zeiss RA 38 microscope equipped with a 100-watt quartz-halogen illuminator, planachromat objectives, 10X compensating eyepieces, 1.3Z Abbe-type condenser, a 3- or 4-mm BG12 primary (exciter) filter, and either a Zeiss No. 50 (500 nm transmission) or a No. 53 secondary (barrier) filter. Smears were scanned using a 16X objective; a 63X dry objective was used to study cell morphologic features. Smears were quantified as: *negative*; *rare*, for 2 to 9 AFB per smear; *few*, for 10 or more AFB per smear; and *numerous*, for more than 2 AFB per 630X field (high dry).

Cultures. All 7H10 and 7H11 media were prepared at this laboratory according to the "Fitzsimons Army Hospital Formula" (2, 6) with the following modifications: the 6 stock solutions were added to 875 ml of deionized water; solution no. 6 was sterilized using membrane filters, and heating was kept to a minimum (7). Drug-free 7H11 plates (8) were inoculated with 6 drops of suspended sediment via Pasteur pipette. Drug susceptibility studies were performed on 7H10 agar plates containing drug; 3 drops of suspension per quadrant were used. Plates were sealed in CO_2-permeable plastic bags and incubated at 36° C in 10 per cent CO_2. Cultures were examined at 3 days for contamination, weekly for 4 weeks, and at 6 and 8 weeks. When the initial smear was positive, a 10-week evaluation was included.

Results

Number of sputum specimens needed to establish culture positivity. Culture histories of 487 patients with culture-positive sputum specimens showed that in 73.7 per cent, the first specimen was positive; a 94.7 per cent positivity rate was achieved by the 6-specimen admission series (table 1). Positive cultures from 26 patients appeared after 7 to 17 sputum evaluations. First specimen positivity rates were lower among patients infected with *Mycobacterium kansasii* or *Mycobacterium avium* complex than among patients infected with *Mycobacterium tuberculosis*, however, these rates equalized with 3 or more specimens. The cumulative positivity rate would have increased by a maximum of only 1.8 per cent, if either a positive smear or a positive culture had been used to denote specimen positivity.

The 455 patients infected with *M. tuberculosis* were grouped according to drug susceptibility and whether they were receiving chemotherapy when admitted to FAMC. The admission positivity rates for 230 patients with drug-susceptible organisms, and not receiving chemotherapy, increased from 76 per cent (first specimen) to 96 per cent (sixth specimen); 136 patients receiving chemotherapy on admission had positivity rates from 73 to 94 per cent. Rates observed among 79 patients with drug-resistant *M. tuberculosis* were 65 to 90 per cent for 40 patients not receiving chemotherapy, and 82 to 92 per cent for 39 patients receiving chemotherapy.

TABLE 1

NUMBER OF ADMISSION SPECIMENS REQUIRED TO ESTABLISH CULTURE POSITIVITY

Specimen Sequence No.	Cumulative Per Cent of Patients Producing Positive Cultures				
	Susceptible *M. tuberculosis* (366 Patients)	Resistant *M. tuberculosis* (79 Patients)	*M. kansasii* (17 Patients)	*M. avium* complex (25 Patients)	All Patients (N = 487)
1	75.1	73.4	58.8	64.0	73.7
2	84.4	79.8	82.4	76.0	83.2
3	88.8	89.9	88.2	84.0	88.7
4	91.3	89.9*	94.1	88.0	91.0
5	94.0	91.4	94.1*	92.0	93.4
6	95.6	91.4*	94.1*	92.0*	94.7
7–17	100.0	100.0	100.0	100.0	100.0

*No additional positive specimens in this category.

TABLE 2

EFFECT OF 4- TO 10-WEEK INCUBATION ON DEMONSTRATION OF MYCOBACTERIA IN SPUTUM SPECIMENS FROM 241 PATIENTS

Patient's Organism	Positive Patients (no.)	Patients with Positive Specimens: All Culture-Positive within 4 Weeks (no.)	(%)	One or More Cultures Negative at 4 Weeks (no.)	(%)
M. tuberculosis (susceptible)	171	126	73.7	45	26.3
M. tuberculosis (resistant)	42	21	50.0	21	50.0
M. kansasii	9	8	88.9	1	11.1
M. avium complex	19	10	52.6	9	47.4
Total	241	165	68.5	76	31.5

Optimal incubation time for positive cultures. Results of a 2-year study of 281 patients are shown in table 2. Isolation plates were incubated at 36° C for 8 to 10 weeks, unless they were positive sooner. All cultures were positive within 4 weeks for 165 patients (68.5 per cent), whereas one or more specimens from 76 patients (31.5 per cent) required 6 to 10 weeks. The slowly growing cultures were isolated more often from patients with drug-resistant *M. tuberculosis* or *M. avium* complex.

Culture data on the effect of prolonging incubation beyond 4 weeks are recorded in table 3. Of 1,950 positive cultures, 91.5 per cent grew within 4 weeks; 8 per cent of the negative cultures (4-week observation) were positive within 6 to 8 weeks. Slower-growing cultures were isolated from patients with drug-resistant *M. tuberculosis;* however, it must be noted that 6.9 per cent of cultures of drug-susceptible *M. tuberculosis* required more than 4 weeks for growth. All specimens from 7 patients required a minimum of 6 weeks of incubation for positivity; furthermore, specimens from 2 patients required 8 weeks.

Agreement of smear and culture results. The reliance on the smear for predicting culture positivity was evaluated from the laboratory data for 270 of the 487 patients (table 4). The cumulative sequential culture positivity rates were essentially the same as those recorded in table 1 for the entire group: 74.1 to 95.6 per cent for the first and sixth specimens, respectively.

Positive smears were 26.7 to 34.1 per cent less effective than cultures at detecting culture-positive patients. For the 6 admission specimens, the mean difference was 31.3 ± 5.4 per cent (2 SD). Approximately one third of the patients might have been misdiagnosed from the smear results alone.

Smear and culture results from 1,620 admission specimens were compared for quantitative and qualitative agreement (table 5). Of 472 culture-negative specimens, 455 were smear-negative. Only 17 specimens were smear-positive and culture-negative, representing 2.4 per cent of the

TABLE 3

EFFECT OF 4- TO 10-WEEK INCUBATION ON DEMONSTRATION OF MYCOBACTERIA IN CULTURES OF SPUTUM SPECIMENS

Patient's Organism	Total No. of Patients	Total No. of Positive Specimens	Per Cent of Specimens Positve Within: 4 Weeks	6 Weeks	8 Weeks	10 Weeks	Specimens Negative at 4 Weeks (no.)	(%)
M. tuberculosis (susceptible)	171	1,288	93.1	5.8	0.9	0.2	90	6.9
M. tuberculosis (resistant)	42	361	83.4	8.6	6.9	1.1	60	16.6
M. kansasii	9	79	98.7	1.3	0.0	0.0	1	1.3
M. avium complex	19	222	93.2	3.5	2.7	0.5	15	6.8
Total	241	1,950	91.5	5.8	2.2	0.4	166	8.5

TABLE 4

NUMBER OF SPUTUM SPECIMENS REQUIRED TO ESTABLISH POSITIVITY VIA SMEAR IN 270 PATIENTS

Specimen Sequence No.	Cumulative Per Cent Patients with		Difference (Culture-Smear) (%)
	Positive Smears	Positive Cultures	
1	47.4	74.1	26.7
2	53.0	83.3	30.4
3	58.2	88.9	30.7
4	59.3	91.5	32.2
5	60.4	94.4	34.1
6	61.9	95.6	33.7
7–17	67.0	100.0	33.0

710 smear-positive specimens. Thirteen smear-positive, culture-negative specimens originated from patients who had received a prior course of chemotherapy or were receiving chemotherapy when admitted to FAMC. In-depth studies of 455 smear-negative, culture-positive specimens revealed that 86.9 per cent produced 1 to 9 colonies, 54.0 per cent with *few*, and 5.8 per cent in the *numerous* category. Quantitative positive smear-culture agreement was seen only in the smear-positive-*numerous* category.

A total of 6,251 sputum specimens from 270 culture-positive patients yielded 2,155 (34.5 per cent) positive cultures and 1,317 (21.1 per cent) positive smears from which 123 smear-positive, culture-negative specimens were recorded. The smear-positive, culture-negative specimens originating from 63 patients (23.3 per cent) represented 2 per cent of the total number of specimens and 9.3 per cent of the total number of positive smears. Seven of 195 patients (3.6 per cent) had smear-positive, culture-negative specimens before initiation of therapy; however, most of smear-positive, culture-negative specimens were obtained within 90 days after therapy was started.

A total of 73 smear-positive, culture-negative specimens were submitted by 63 patients: 8 were collected before therapy, 50 were interspersed among culture-positive specimens or occurred fewer than 60 days after the last positive culture, and 15 were seen 60 or more days from the last positive culture. One smear-positive, culture-negative specimen was directly associated with subsequent reversion to bacteriologic positivity.

Contamination rates. The primary contaminant in this laboratory is an aerobic sporeformer with rapid agar surface growth characteristics. The contamination rate data are depicted in table 6. In-house control measures, including better housekeeping, control of air movements, and the use of pre-dried plates, decreased the rate to the desired 1 to 5 per cent.

Discussion

The proportion of patients infected with drug-susceptible *M. tuberculosis* that was detected by 6 admission culture specimens has varied little since 1968. The occasional overnight storage of specimens at 4° C did not affect the recovery of *M. tuberculosis;* these observations are in agreement with reports that few *M. tuberculosis* cells are lost at 4° C, even after 1 week (9). The optimal number of consecutive sputum specimens for detecting culture-positive patients was 5 (table 1); this finding is in agreement with published data (10).

In this laboratory, the efficacy of smears and cultures at detecting active tuberculosis was better than some reports suggested (11, 12); however, a reported 90 per cent success rate with only 2 specimens exceeded our results (13). Patient differences may account for these contradictions; the group in the present study con-

TABLE 5

AGREEMENT OF SMEAR AND CULTURE RESULTS OF 6 ADMISSION SPUTUM SPECIMENS FROM 270 CULTURE-POSITIVE PATIENTS

Culture Results	Positive Smears								Negative Smears		Total Specimens	
	Rare		Few		Numerous		Total					
	(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)
1–9 colonies	25	23	12	7	4	1	41	6	273	60	314	27
Few colonies	52	47	62	35	14	3	128	18	150	33	278	24
Numerous colonies	34	30	103	58	387	96	524	76	32	7	556	48
Total no. positive	111		177		405		693		455		1,148	
Negative	10		5		2		17		455		472	
Total no. specimens	121	7	182	11	407	25	710	44	910	56	1,620	100

TABLE 6

CULTURE CONTAMINATION RATES FROM 1969 TO 1973 FOR 16,482 TUBERCULOSIS WARD SPUTUM SPECIMENS

Year	Range* (%)	Mean (%)	SD (%)	Median (%)
1969	3.7–17.9	9.8	4.7	9.8
1970	0.8–9.4	4.2	2.8	2.9
1971	0.5–7.1	2.8	2.2	1.6
1972	0.0–5.8	2.6	1.6	2.2
1973	1.1–6.6	3.9	1.9	3.6

*Monthly rates. Final sodium hydroxide concentration: September 1969, increased to 3.0 per cent; July 1970, decreased to 2.5 per cent; June 1971, decreased to 2.0 per cent.

sisted primarily of well-nourished, young adult men.

Discarding growth-negative cultures after a 4-week incubation would have eliminated 8.5 per cent of positive cultures (table 3). Longer incubation times are necessary for drug-resistant bacilli, and especially for *Mycobacterium xenopi* and *Mycobacterium ulcerans*. Cell damage from exposure to alkali decontamination or *in situ* metabolic activity of drug(s) probably contributes to the recorded slow growth characteristics of all species.

Studies on the growth responses of *M. tuberculosis* on such media as Gruft (14), Mycobactosel-Löwenstein-Jensen, Mycobactosel-7H11 (15), Mitchison (16), Löwenstein-Jensen, and 7H11 medium, showed that 7H11 and Löwenstein-Jensen media compared favorably; Gruft was less efficacious than drug-free 7H11 or Löwenstein-Jensen medium; Mycobactosel formulations were the least suitable, and excellent recovery of *M. tuberculosis* from raw sputum was obtained using the Mitchison medium. In all cases, growth was better on drug-free medium than on medium containing drug.

Smears were poor substitutes for cultures in detecting culture-positive patients (table 4); these findings are in agreement with other reports (17, 18). A 31 per cent lower positive patient rate was detected by smear evaluation alone. The maximal recorded colony counts from 270 positive patients were as follows: less than 10 colonies from 47 patients, few (10 to 99) colonies from 100 patients, and numerous (100 or more) colonies from 108 patients. One can speculate that the negative smear findings associated with the observed sparse growth (table 5) were due to paucity of AFB. Sixty per cent of the smear-negative, culture-positive category was attributed to specimens with 1 to 9 colonies.

The smear-positive, culture-negative findings may be attributed to the following causes: the patient was in the process of reverting from negative to positive sputum status; the laboratory erred, or it was a normal occurrence in the course of therapy. A 3.6 per cent smear-positive, culture-negative rate was recorded for specimens from 270 untreated patients; this rate was lower than the 6 to 7 per cent rate reported at Fitzsimons in 1955 (19); however, the 2 populations were not strictly comparable. Investigators have shown that the occurrence of smear-positive, culture-negative specimens parallels the period of maximal elimination of bacilli, which is 60 to 90 days after chemotherapy is begun (19–22).

Therapeutic regimens of rifampin and/or pyrazinamide and regimens of rifampin and/or isoniazid appear to increase the incidence of smear-positive, culture-negative specimens (20, 22). Our data support these findings; however, more smear-positive, culture-negative specimens were seen in patients on isoniazid regimens than in those receiving rifampin. Isolated smear-positive, culture-negative specimens are difficult to explain and have been reported "less likely [to] represent tubercle bacilli or be of clinical significance" (21). In one study, 70 per cent of acid-fast colonies isolated 6 months or more after sputum conversion were recorded as mycobacteria other than *M. tuberculosis* (23).

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