A Study on the New Slide Culture Method

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Previously, I have published a few studies (1, 2) on the improvement of the slide culture method. I have since been able to introduce greater improvements in the slide culture method, both on the early detection of tubercle bacilli in sputum and on the screening test of tuberculostatic substances. The new methods developed will be reported in this paper.

Chapter I. A New Slide Culture Method employing Benzine-treated Bacillary Suspension

Introduction

For the estimation of the bacteriostatic power of chemicals on the tubercle bacillus in vitro, methods have been used mainly, in the past to determine whether or not growth of tubercle bacilli is optically detectable in a liquid or solid medium containing a certain density of agent.

There is, however, in these optical methods the great disadvantage that a long period is required for culture, and it is impossible to prevent changes of the agents contained in the medium during the aging process of a culture. Therefore the Slide Cell Culture (S. C. C.) or Slide Culture Method (S. C. M.) (3, 4), by which the increase in growth of tubercle bacilli can be demonstrated microscopically within seven days, have an advantage over the above mentioned methods in which the growth is optically shown within three or four weeks.

But as is known, the S. C. C. has an unavoidable difficulty in the determination of experimental results and the old S. C. M. also has an unavoidable disadvantage in that the tubercle bacilli smeared on a slide from the

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storage strains suspended in aqua pura or physiological saline solution, are apt to fall off the slide during the experiment.

Moreover, in both of these methods there is the possibility of misinterpreting the results because of the difficulty of obtaining a strictly single cell suspension even after many hours and much work. Thus it seems that these slide methods are also not yet satisfactory for the purpose of determining the effect of tuberculostatic agents in vitro.

However, the study on the removal of the cord factor of the tubercle bacillus, discribed by H. Bloch (5) in 1950, suggested to me the application of petrol ether in a new S. C. M. in which a loopful of petrol ether-treated bacillary suspension is placed on a slide and fixed by drying. Nevertheless, from the results of the experiments described below, petrol benzine has proved preferable to petrol ether.

In this case, tubercle bacilli on a smear slide are perfectly separated individually and are fixed satisfactorily on the slide during a culture. Moreover, most of single bacterial cells grow on the slide forming characteristic serpentine colonies.

This new slide culture method is believed to be the most suitable for the estimation of the bacteriostatic power of chemicals on the tubercle bacillus in vitro.

Preliminary Experiment

An investigation on the dispersion of tubercle bacilli in different suspensions treated with petrol ether as used by H. Bloch or with related solvents was attempted: a mass of tubercle bacilli taken from the surface pellicles of Sauton's liquid medium was put in each glass tube. Approximately 2 cc of each solvent was added and the tube plugged. The glass tubes were shaken by hand for a few minutes and were centrifuged for five minutes at 1000 r. p. m.. Then the degrees of dispersion of tubercle bacilli in each suspension were compared by Ziehl-Neelsen's stain smears of a loopful of each supernatant fluid.

Under the same conditions, the degree of dispersion of tubercle bacilli on each smear slide varied considerably according to the solvent used, though the tubercle bacilli were sharply separated in every case. The count of bacilli ranged in the following order: ether, chloroform, xylene, toluene, benzine, petrol ether, and petrol benzine.

Another investigation was made on the injury to the tubercle bacillus caused by the different solvents. A loopful of each bacillary suspension was incubated into Oka-Katakura's solid medium and a loopful of each was also smeared on slides to be allowed to grow by the new S. C. M. as exp-

lained in this paper. After incubation for a certain period, the colony counts of the cultures were compared.

Table 1 summarizes the results.

Table I. Comparison of the Injury of the Different Solventes to Tubercle Bacillus

Time	Benzine	Petrol Ether	Toluene	Xylene	Ether	Chloroform
10′	++ (++)	++ (++)	- (-)	- (-)	- (-)	- (-)
30′	++ (++)	++ (++)	- (-)	- (-)	- (-)	- (-)
1°	++ (++)	++ (++)	- (-)	- (-)	- (-)	- (-)
2°	++ (++)	++ (++)	- (-)	- (-)	- (-)	- (-)
3°	++ (++)	++ (++)	- (-)	- (-)	- (-)	- (-)
5°	++ (++)	++ (++)	- (-)	- (-)	- (-)	- (-)
24°	++ (++)	++ (+)	- (-)	- (-)	- (-)	- (-)
48°	++ (++)	++ (++)	- (-)	- (-)	- (-)	- (-)

( ); The results of the new S. C. M.

Of the solvents used in the experiments, petrol benzine proved best for the S. C. M. The degrees of dispersion of these suspensions are different according to the strains and the ages of a culture. The tubercle bacilli are individually dispersed more easily in the bacillary suspension of a young culture than of an old culture. Of strains tested, strain H_2, Aoyama B, and BCG are best suspended in petrol benzine, bovine strain RM and Bovine 1. are fairly well suspended, strain F is a little inferior and the avian strain is the worst. When the bacillary suspension becomes uniformly dispersed only with difficulty, dispersion can be somewhat accelerated by the addition of a drop or more distilled water, or further by warming it with the hands. This is generally observed in an old culture or with dry tubercle bacilli and therefore the tubercle bacillus of a 1 to 2 week culture seems to be most suitable for the S. C. M.. In this new method, the state of bacillary fixation on the slide was sufficient even during a culture.

Method

I first performed the new S. C. M. by a modification of the slide culture method described by Berry and Lowry and developed by Yonezu, who smear slides from a specimen of sputum.

The technique is carried out as follows;

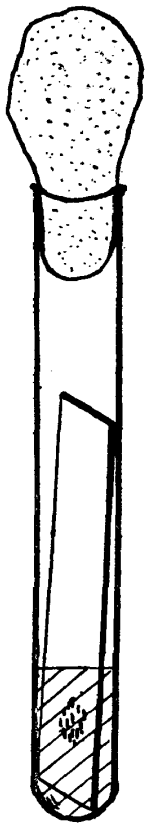
1. A loopful or more of tubercle bacilli from the surface pellicles on a liquid or solid medium is put in a glass tube and 2 cc of petrol benzine is added.

2. The glass tube is plugged and shaken by hand for a few minutes and then centrifuged for few minutes at about 1000 r. p. m.

3. A loopful of the supernatant fluid is placed on the end of sterile slides (made by cutting a normal microslide logitudinally into two pieces) in Petri dishes. The benzine alone promptly drys by evaporation.

4. Employing sterile slide forceps, the smears are put into the normal sized or shorter glass tubes (one slide per tube) containing 5 cc of Kirchner's or Youmans' liquid medium containing a certain dose of tuberculostatic agent. Then the tubes are placed vertically in a tube stand after plugging with cotton plugs, as shown in figure 1..

Fig. I



5. After incubation at 37°C for a few days, a smear is removed from a control tube (no tuberculostatic agent) and stained by the usual Ziehl-Neelsen techniques. The preparation is examined under low power and if colonies with serpentine cords are seen, the remainder of the smears are also removed, stained and examined in a similar manner. The concentration of tuberculostatic agents which inhibit growth of tubercle bacilli on the slide is considered the end point.

However, in the above mentioned method, about 5 cc of liquid medium is necessary at least. Therefore, a further improvement has been introduced in the S. C. M. in order to save on medium. The new technique of the S. C. M. needs only 1 cc of liquid medium.

The technique is carried out as follows;

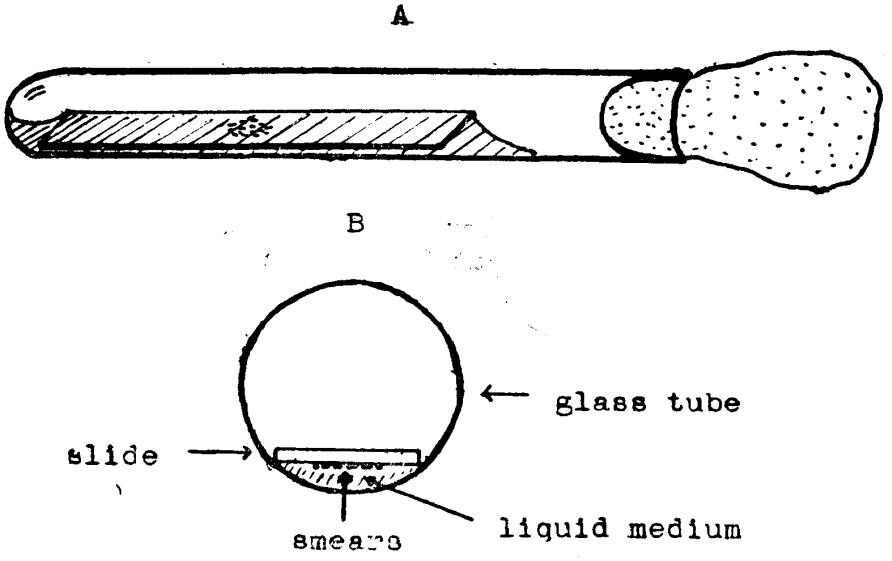
1.-2. Similar to the above mentioned methods.

3. A loopful of the supernatant fluid is placed on the slide made by cutting a normal microslide longitudinally into three or four pieces instead of the slide made by cutting it longitudinally into two pieces.

4. The smear is placed in the normal sized glass tube containing 1 or 0.5 cc of Kirchner's or Youmans' liquid medium. After plugging the tube with cotton and paraffin plug or rubber plug in order to preserve the liquid medium from evaporation, it is placed horizontally in a tube stand laid on its side as shown in figure 2.. The smeared side of the slide is placed downwards in the glass tube so that it is immersed in the liquid medium. The surface tension of the liquid medium prevents the medium from reaching the plug when the tube is laid horizontally.

5. After incubation at 37°C for 3 or 4 days, a smear is removed from a control tube and stained by the usual Ziehl-Neelsen technique. The preparation is examined under high power and if bundle forming colonies consisting of several bacilli are seen, the remainder of the smears are also removed, stained and examined in a similar manner.

Fig. II



Employing this new S. C. M., the estimation of the bacteriostatic power of various tuberculostatic agents on the tubercle bacillus is performed.

The results are summarized in table 2..

Table II Conparison of the Effect of the Tuberculostatic Agents estimated by the New S. C. M.

Agents	Strains	Dilution Degree (thousand) 160	320	640	1280	2560	Control
Streptomycin	H_2	−	−	−	−	++	++
	F	−	−	−	−	++	++
	Aoyama B	−	−	−	−	++	++
	Sputum G. 3.	−	−	−	++	++	++
PAS	H_2	−	−	−	+		++
	F	−	−	−	+		++
	Aoyama B	−	−	−	+		++
	Sputum G. 3.	−	−	−	++		++
O-Aminophenol	H_2	−	−	−	++		++
	F	−	−	−	+		++
	Aoyama B	−	−	−	++		++
	Sputum G. 3.	−	−	−	++		++
Usnic acid natr.	H_2	−	−	−	++		++
	F	−	−	−	++		++
	Aoyoma B	−	−	−	++		++
	Sputum G. 3.	−	−	−	++		++

Chapter II A New Slide Culture Method employing a Sputum Concentrate treated with 0.5 per cent KOH Solution

For the demonstration of the presence of tubercle bacilli, or their resistance to Streptomycin, in a specimen of sputum, the direct smear preparation has generally been utilized in the S. C. M. or its modifications reported

in the past. These techniques are very troublesome and there are frequently an insufficient number, less than Gaffky O, for demonstration.

This fact led me to the application of the NaOH solution-treated sputum concentrate for the S. C. M.. The sputum is treated with 4% NaOH solution and it is centrifuged at 3000 r. p. m.. The sediment is washed with sterile water and it is again centrifuged at 3000 r. p. m.. A loopful of the sputum concentrate is in turn placed on the slide. By the above mentioned manner, tubercle bacilli could be allowed to grow on the slide. The proportion of the detection of organisms in sputum has considerably increased but the contamination rate also has become higher because the material is centrifuged twice in this technique. If the process of washing the sediment with sterile water is omitted, the smear on a slide falls off the slide during a culture.

However, by chance, I have found that smears of KOH solution-treated sputum concentrate, do not fall off the slide, not only during the process of staining but also when the smear is submerged in liquid media for a long time. Moreover, KOH is stronger in chemical action than NaOH and the specific gravity of the former solution is a little lower than the latter in the same density. In consideration of these facts, an investigation was made to determine which percent of KOH solution could be utilized, not only for concentration but also for destroying organisms other than the tubercle bacillus.

Preliminary Experiment

Specimens of mucous sputum, purulent sputum, and various other kinds of sputum from several tuberculous patients, are mixed together in a mortar and are ground into a uniform substance. And then 1 cc of the specimen is placed in each of several graduated tubes and 10 cc of KOH or NaOH solutions of different percent are added. After shaking the glass tubes by hand for about 5 minutes until the specimens are homogeneous, they are centrifuged at 3000 r. p. m. for 15 minutes. The degree of concentration is indicated by the graduation on the glass tubes.

The results are summarized as follows;

KOH:- 1. The measure of centrifuged sediment of sputa treated by 0.5 or more percent KOH solution amounted to about 0.02–0.05 cc.. The quantity of the sediment did not vary according to the percentage of KOH.

2. Sediment of sputum treated with 0.1% KOH solution amounted to 0.3–0.15 cc.. In this case, the amount of sediment increased.

3. Sediments of sputum treated with 0.4–0.2% KOH solution fell within a wide range: 0.2–0.3 cc.

NaOH:- The amount of centrifuged sediment of sputa treated with 1% NaOH solution was equivalent to that from sputum treated with 0.5% KOH solution.

A loopful of each sputum concentrate treated with the different per cent of KOH solution was inoculated into glass tubes containing 5 cc of bouillon. After incubation at 37°C for 48 hours, the presence or absence of bacteria was determined by comparing their turbidity with a control, to which no sputum concentrate had been added.

The results are summarized in table 3..

Table III Contamination Rate of KOH-, NaOH-, or H_2SO_4-Solution

Agents	Degree of Solution	Time of Treatment 5′	15′	30′
NaOH	0.5	+	+	+
	1.0	+	+	+
	2.0	+	±	-
KOH	0.5	+	±	-
	1.0	+	-	-
	2.0	-	-	-
H_2SO_4	0.1	-	-	-
	0.2	-	-	-
	0.3	-	-	-

In the experiment, it was found that bouillon inoculated with the sputum concentrate treated by 0.5% KOH solution for 30 minutes or by 1% KOH solution for 20 minutes are never contaminated. But, in order to save time, furacin was dissolved in the KOH solution.

The action of furacin on tubercle bacilli was tested by the above mentioned method employing benzine.

The results are summarized as follows;

Action of Furacin on Tubercle Bacillus

Density of Furacin	1/10000	1/20000	1/40000	1/80000	1/160000	1/320000	Control (no Furacin)
Growth of Tubc. Ba.	+	+	++	++	++	++	++

Action of 1% KOH solution on tubercle bacillus was tested by the following technique;

Tubercle bacilli from surface pellicles of Sauton's liquid medium were suspended in water according to the usual technique and 1 cc of this bacillary suspension was placed in each of three glass tubes, to which 10 cc of 1% KOH solution, 4% H_2SO_4 solution, and sterile water was added respectively. After treatment for 30 minutes, 1, 2, 3, or 5 hours, a loopful of each solution was incubated on Oka-Katakura's solid medium. After incu-

bation at 37°C for thirty days the number of colonies was counted. Generally, colonies were found earlier and grew more abundantly in the following order: sterile water, KOH solution and H_2SO_4 solution.

Thus it is found that 0.5% KOH solution containing 0.0005 per cent furacin is most suitable in preparing sputum both for cultivation and for concentration. An additional advantage is that a film prepared from a KOH-treated concentrate does not fall off the slide.

However, when a specimen of sputum is treated with 2 per cent or stronger KOH solution the concentrate film on the slide sometimes does fall off, moreover, the action of furacin is reduced in higher alkaline solutions.

In consideration of these facts, the new technique was designed.

Method

1. The desired quantity (usually 1–3 cc) of a specimen of sputum is placed in a centrifuge glass tube and 5 times its volume of 1 per cent KOH solution is added. The mixture is shaken by hand until the sputum is homogeneous about 5 minutes.

2. An equal quantity of 0.001 per cent furacin solution is added and shaken by hand for a few minutes.

3. After centrifuging for 15 minutes, a loopful of the concentrate is smeared on the above-described slide and the smear is dried in an incubator.

4. The preparation is placed in an ordinary glass tube containing 1 cc of Kirchner's or Youmans' liquid medium in which 0.0005% of malachit green is added and is incubated at 37°C according to the above-mentioned technique, in which the tube is laid horizontally in the incubator.

5. On the fourth, seventh, and tenth days, the slides are removed from the tubes and allowed to dry, fixed, labeled with heat, stained and examined under low power.

The results of the demonstration of tubercle bacilli in sputum treated by this technique are summarized in table 4..

Table IV Detection Rate of Tubercle Bcilli by Both Culture Method

Culture Method	S.C.M. (by weak power)			O.K. solid medium	
Incubation Days	4	7	10	30	60
Positive	3	13	15	10	11

Of cases negative by concentration technique

In the experimental results, the rate of demonstration of tubercle bacilli in a specimen of sputum is higher than in the usual method employing solid media.

This method can also be utilized as a sensitivity test in determining the effect of tuberculostatic agents, and the resistance of tubercle bacilli.

Conclusion

A new slide culture method employing a benzine-treated bacillary suspension is a rapid, easily performed screening test.

A new slide culture method employing sputum concentrate treated with 0.5 per cent KOH solution is a rapid, easily performed technique for the detection of tubercle bacilli in specimens of sputum, and is also a useful sensitivity test in measuring the effectiveness of tuberculostatic agents and the resistance of tubercle bacilli to them.

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