PLATE METHODS FOR TESTING ANTIBIOTIC ACTIVITY OF ACTINOMYCETES AGAINST VIRULENT HUMAN TYPE TUBERCLE BACILLI[1]

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A simple way to test the antibiotic properties of an organism is to streak it on an agar plate and then, after growth has been established, cross-streak with the organism against which it is to be tested (Waksman, 1945). The zone of inhibition of the test organism can then be measured. Thus, by cross-streaking with many organisms, it becomes fairly easy to establish a "spectrum" of the inhibiting properties of any bacterium, mold, or actinomycete which will grow discretely on an agar plate.

Once the bacteriostatic properties of an agent have been established, there are many ways of testing it quantitatively. Extracts and filtrates of the culture of the effective organism may be tested by serial broth dilutions, turbidimetric measurements, agar plate dilutions, and cylinder plate methods (Waksman, 1945). Animal tests may supplement these methods after a nontoxic extract or filtrate has been prepared.

Serial dilution methods and animal tests have been useful in measuring the reaction of antibiotic extracts on virulent human type tubercle bacilli. Bush, Dickinson, Ward, and Avery (1945) report the use of the cylinder plate method with the rapidly growing nonpathogenic strain of tubercle bacillus known as "607," but slowness of growth and difficulties in preparing suspensions of virulent human type tubercle bacilli are probably responsible for the fact that the cross-streak and the cylinder plate method have not, to the knowledge of the authors, been reported using these organisms.

This paper reports three agar plate methods which have been used to select actinomycetes with antibiotic properties and to test quantitatively filtrates and concentrates derived from these.

METHODS AND RESULTS

Cross-streak method. Thirteen strains of actinomycetes[2] were selected for the tests. Seven media were chosen which would promote the growth of the actinomycetes and to each of these were added glycerol to the amount of 2 per cent and agar to the amount of 1.5 per cent. The glycerol may be omitted but the growth of tubercle bacilli is slower. The complete formulae for these media follow:[3]

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[2] Obtained from Dr. John Ehrlich of Parke, Davis and Company, Detroit, Michigan.

[3] Formulae for these media were furnished by Dr. John Ehrlich, Parke, Davis and Company, Detroit, Michigan.

Medium 1

Corn steep liquor (Corn Prod. Ref.)	1.0%
K_2HPO_4	0.2%
NaCl	0.5%
Cerelose (Corn Prod. Ref.)	1.0%

Medium 2

Corn steep liquor	1.0%
K_2HPO_4	0.2%
NaCl	0.5%
Maltose, tech. (Difco)	1.0%

Medium 3

Curbay B-G (U. S. Indus. Chem.)	0.5%
Casamino acids (Difco)	0.5%
NaCl	0.5%
Cerelose	1.0%

Medium 4

B-Y fermentation solubles (Comm. Solv. Corp.)	0.5%
Casamino acids	0.5%
NaCl	0.5%
Cerelose	1.0%

Medium 5

Beef extract (Difco)	0.3%
Peptone (Difco)	0.5%
Maltose	1.0%

Medium 6

Corn steep liquor	1.0%
K_2HPO_4	0.2%
NaCl	0.5%
Maltose cp	1.0%

Medium 7

Beef extract	0.3%
Peptone (Difco)	0.5%
NaCl	0.5%
Glucose	1.0%

These media were adjusted to a pH of 7.0, tubed in 40-ml amounts, and stored in the icebox until needed. When melted and poured into plates this amount of medium helped to provide for loss by evaporation. The actinomycetes were streaked on the agar plates with a 4-mm loop from a spore suspension made by pouring saline over a sporulating slant and loosening the spores with a loop, or directly from a more stable preparation made by mixing the spores in a gelatin suspension and drying. These plates were incubated at 24 C for 5 days, or until a streak of growth about 1 cm in width had been established.

A thick suspension of the H37Rv strain of *Mycobacterium tuberculosis* was obtained by grinding a 14- to 21-day-old pellicle growth from a flask of Proskauer

and Beck synthetic medium and diluting with 0.01 molar phosphate buffer solution until a thick, pasty preparation was obtained. This thick, pasty inoculum was necessary to give uniform streak growth.

Streaks of H37Rv were made with a 4-mm loop at right angles to the actinomycete streak, and the plates incubated at 37 C. The growth of tubercle bacilli was at a maximum in 2 to 3 weeks and appeared as a wide rugose band 2 or 3 times the width of the original inoculating loop. The degree of inhibition of the tubercle bacillus was measured in millimeters from the edge of the actinomycete streak. Where several streaks of the same strain of tubercle bacillus were made, the readings were averaged. Several plates were also streaked with both H37Rv and H37RvR, the latter a strain of H37Rv which had been made resistant *in vitro* to more than 1,000 micrograms of streptomycin per ml of medium (Williston and Youmans, 1947). Figure 1, nos. 1, 2, and 3, show results of cross-streaking actinomycete plate cultures with H37Rv and H37RvR.

At the time the results were observed, the hydrogen ion concentration of the agar adjacent to the streak was determined in order to eliminate inhibition due to acidity alone. The hydrogen ion concentration was determined by cutting out strips of the agar and dissolving them in distilled water in the cup of a Coleman electrometer. Table 1 shows the width of the zone of inhibition (in mm) of the H37Rv strain of tubercle bacillus by 14 strains of actinomycetes on seven different media chosen because they favored growth and antibiotic production by the actinomycetes. Table 2 shows the results obtained on two media comparing the degree of inhibition of growth produced by the actinomycetes on the virulent H37Rv and the avirulent 607 strain. Not only are the organisms inhibited to a different degree, but several actinomycetes inhibited the virulent H37Rv and not the avirulent 607 strain. Obviously, if only the avirulent strain were used in these tests, effective antibiotics might be missed.

Cylinder plate method. This method was used in an attempt to make quantitative studies on filtrates and extracts of cultures which had already shown inhibitory properties. The medium used was a modified Proskauer and Beck synthetic medium to which was added 1.5 per cent agar. Forty ml of the nutrient agar were first put in the plate and allowed to harden, and then a 4-ml quantity of the agar that had been seeded with 7.5 mg (Hopkins tube) of tubercle bacilli per ml of agar was poured over the surface. This inoculum of tubercle bacilli for the seeded layer was ground with mortar and pestle until very smooth so that the opacity of the growth layer was uniform after incubation. Stainless steel cylinders were dropped gently through a plastic "guide" onto the plates. Into these cylinders were delivered the diluted extracts or filtrates. The plates were incubated 2 or 3 weeks and the diameters of the zones of inhibition were measured in mm. Similar pour plates were also made using the streptomycin-resistant strain of H37RvR. The cylinders were refilled when necessary, from time to time, with the extracts or filtrates to replace loss of potency due to exposure at incubator temperature.

Cylinder plates were made using four cylinders to a plate. Two of the cylinders on each plate contained 10 and 5 micrograms, respectively, of strep-

tomycin per ml. These consistently gave zones of inhibition of tubercle bacilli of approximately 25 and 15 mm, respectively, and served as controls. Filtrates and concentrates of the antibiotics to be tested were placed in two different

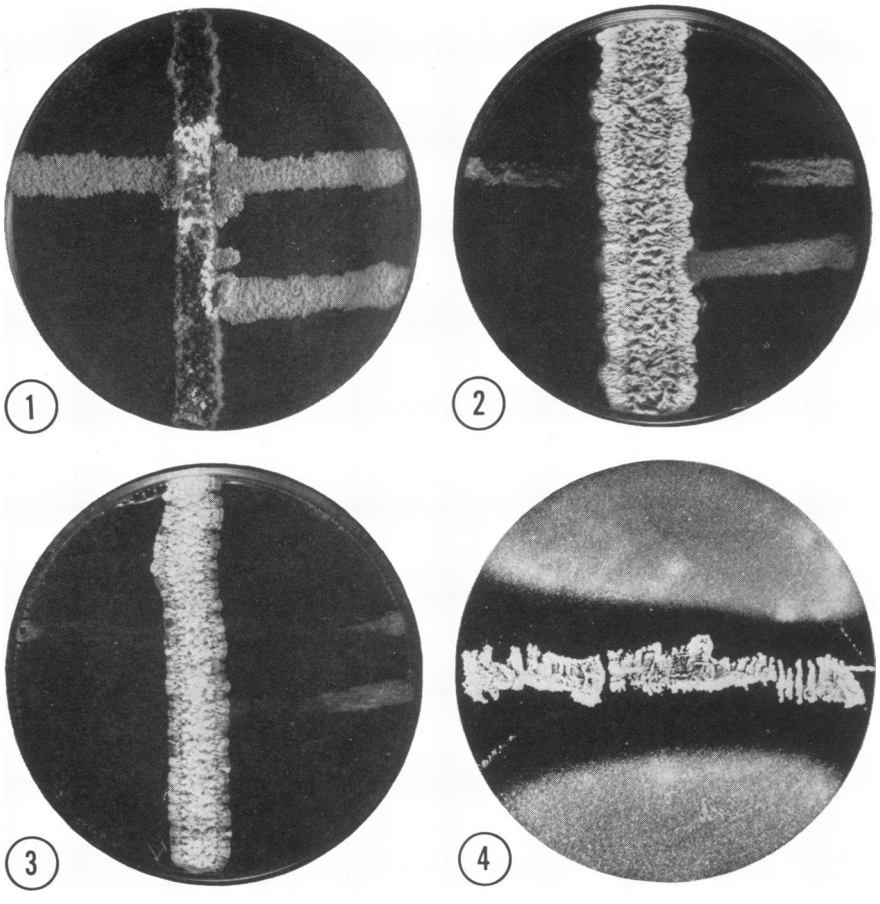

FIG. 1. ACTINOMYCETE CROSS-STREAKED WITH TUBERCLE BACILLUS
Vertical streak: Actinomycete. Upper horizontal streak: H37Rv. Lower horizontal streak: H37RvR (resistant to >1,000 micrograms streptomycin).
No. 1. No inhibition of either streptomycin-sensitive or streptomycin-resistant tubercle bacilli.
No. 2. Inhibition of streptomycin-sensitive strain only.
No. 3. Inhibition of both streptomycin-sensitive and streptomycin-resistant strains.
No. 4. "Streak pour plate" seeded with H37Rv and cross-streaked with an inhibitory organism.

dilutions in the other cylinders. A comparison with streptomycin could thus be established.

Since the margins of the zones of inhibition were usually very fuzzy and indistinct, quantitative data were difficult to obtain. In some cases, however, clear-cut zones were noted.

Streak plates seeded with tubercle bacilli. The plates seeded with tubercle

TABLE 1

*Plate cultures of actinomycetes cross-streaked with virulent human type tubercle bacilli H37Rv and H37RvR**

ACTINOMYCETE CULTURE NO.	AMOUNT OF INHIBITION IN MILLIMETERS							
	Medium							
	1	2		3	4	5	6	7
	H37Rv	H37Rv	H37RvR*	H37Rv	H37Rv	H37Rv	H37Rv	H37Rv
1	>37	25	25	6	3	13	>32	
2	17	15	18	0	0	1	21	
3	20	0	0	6	7	1.5	17	
4	27	9.2	0	11	10	11	27	
5	14	11.3	6.3	11.3	0	7	15	
6	20	16	17.5	11	0	10	17	20
7	13	2.5	4	0	0	2	14	
8	0	0	0	0	0	23	0	0
9	17	19.6	0	8	10	15	15	
10	16	18	15	0	26	3	18	
11	0	0	0	0	0	0	0	0
12	20	3	4	10.6	11	8.3	12	
13	15	15	20	12.5	11.6	18	14	15
S. griseus†	20	12	0			12	20	0

* Resistant to streptomycin.

† Furnished through the courtesy of Dr. Selman A. Waksman, New Brunswick, New Jersey.

TABLE 2

Comparison of streak test results obtained with H37Rv and 607

ACTINOMYCETE CULTURE NO.	AMOUNT OF INHIBITION IN MILLIMETERS			
	Medium 6		Medium 1	
	H37Rv	607	H37Rv	607
1	>32	7	>37	13
2	21.3	16	17.1	11.2
3	17.3	16	20.2	10.7
4	27	11.2	27	13.5
5	15	6	14	6.5
6	17	17	20	13
7	14	0	13	0
8	9	0	0	0
9	15	20	17	20.7
10	18.2	0	15.8	0
11	0	0	0	0
12	12	0	20	0
13	20.4	18.5	19.2	15
S. griseus	20.2	21.5	20.5	?

bacilli prepared as described above were also used for streaking the actinomycete cultures. These were incubated first at 24 C for 5 days, then at 37 C for 2 weeks.

If any of the actinomycetes possessed bacteriostatic properties, a zone of inhibition of the tubercle bacilli growing in the agar appeared next to the streak.

Pour plates were seeded with both the resistant H37Rv and the sensitive strain. Eight of the actinomycetes were cross-streaked and the inhibition zones measured. These inhibition zones were approximately the same as those obtained by cross-streaking the actinomycete with tubercle bacilli, as recorded in table 2. Figure 1, no. 4, shows an inhibitory organism cross-streaked on a pour plate seeded with H37Rv.

CONCLUSIONS

The streak plate method using the virulent type of tubercle bacillus (H37Rv) is useful for the testing of the antibiotic properties of actinomycetes. This gives a relatively rapid method for screening cultures in a search for new antibiotics. If a streptomycin-resistant strain of H37Rv is also streaked on the plates, cultures bearing a relationship to *Streptomyces griseus* may be detected.

A smooth, opaque layer of growth may be obtained by seeding pour plates with H37Rv. Filtrates and concentrates in cups will give inhibition zones, though quantitative measurements are difficult to make because the zones are not always sharply defined.

Pour plates, seeded with tubercle bacilli and streaked with actinomycetes, are useful in the search for cultures with tuberculostatic properties. The plates may be seeded with H37Rv or with H37RvR (resistant to streptomycin) and cross-streaked with various strains of actinomycetes.

The avirulent, rapidly growing strain 607 is not suitable for this purpose, since some strains of actinomycetes which inhibit the virulent H37Rv strain do not inhibit, under the same conditions, strain 607.

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