AN AGAR DIFFUSION METHOD FOR EVALUATION OF ANTIMYCOPLASMA SUBSTANCES

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An agar diffusion method for testing antibiotics and other chemicals inhibiting *Mycoplasma* species has been developed. Paper discs dipped in solutions of the candidate compounds are placed on agar plates heavily seeded with *Mycoplasma* cells. After over-night incubation, the agar surface is flooded for 5 minutes with a 2, 6-dichlorophenolindophenol solution. The viable cells reduce the dye while the killed cells do not. Dose response curves with slopes between 3 and 4 were obtained when discs containing chloramphenicol, chlortetracycline, or tylosin were placed on agar seeded with *Mycoplasma laidlawii* type B. The technique can also be used for preparing bioautographs of paper or thin layer chromatograms.

With the increasing interest in the use of antibiotics in the control of *Mycoplasma* infections in man[1] and animals[2], there is a need for rapid methods for determining the effectiveness of a variety of antibiotics and other medicinal chemicals in inhibiting *Mycoplasma* cultures. Heretofore, the methods used included tube dilution assays[3,4] and agar dilution methods, *e.g.* where the candidate compound is diluted in agar solutions and the surface of the agar is streaked with the test organisms and observed for growth (using a microscope) after incubation[5,6]. We have found that suspensions of *Mycoplasma* cells will reduce 2, 6-dichlorophenolindophenol and have used this capability as the basis of a visual endpoint for an agar diffusion assay for antimycoplasma substances.

Materials and Methods

The *Mycoplasma laidlawii* type B cultures used in this study were maintained in soypeptone broth supplemented with yeast extract, NaCl, and human serum as mentioned by KENNY and POLLOCK[7] and this solution solidified with Noble agar. In the assay a 10 ml layer of the soypeptone agar is poured into a 150 mm Petri dish and allowed to harden. Five ml of the same agar inoculated with about 10^8 cells from a 24 to 40 hr old culture is spread on the surface of the agar base layer. When this layer is hardened 6.35 mm filter paper discs* dipped in the test solutions are placed on the agar surface. The Petri dishes are incubated over night in a 37°C. Following this incubation period, the discs are removed and the agar surface flooded for 5 minutes with a 0.05 % aqueous 2, 6-dichlorophenolindophenol solution. After removal of the dye, the plates are returned to the incubator for 60 to 90 minutes. The dye stains the areas where the cells have been killed by the anti-mycoplasma solution, while those areas of the plate where the cells are still viable are colorless.

* Schleicher and Schuell Co. No. 740-E with holding capacity=0.03 ml H_2O.

The antibiotics used in this study were obtained from commercial sources and were not purified further prior to use.

Results

Observations on the inhibition of *M. laidlawii* type B in this test system are summarized in Table 1. Rather large zones of inhibition were obtained with only 3 mcg/disc, and it is likely that useful information will be realized for most antibiotics with only 0.3 mcg/disc. Data obtained in testing a series of dilutions of tylosin, chloramphenicol, and chlortetracycline are summarized in Table 2. When these are plotted on semi-log paper the dose-response curves have slopes between 3 and 4.

The methods used for the above assay were applied to bioautography of paper chromatograms using a 3-quart Pyrex baking dish with 150 ml of base agar and 80 ml of seed agar. The paper chromatogram is placed on the agar surface for 30 minutes and then removed. After over-night incubation at 37°C the dye solution is added to the agar surface for 5 minutes and then poured off. The plate is then returned to the incubator for 1 hour. Very clear zones of inhibition are usually found represented by blue-stained areas against the clear background. Similar results are obtained (as far as the bioautograph is concerned) with thin layer chromatograms.

Table 1. Agar diffusion bioassay for antimycoplasma substances using *M. laidlawii* type B

Compound tested	Diameter of inhibition zone, mm	
	30 mcg/disc	3 mcg/disc
Actinospectacin	26.5	17.5
Erythromycin	36	30.5
Gentamicin	30.5	24
Kanamycin	26	19.5
Neomycin	26	20
Novobiocin	17	14
Paromomycin	25 (diffuse edge)	16 (diffuse edge)
Penicillin G	no zone	no zone
Sodium pyridinethione	26	14
Spiramycin	28.5	23.5
Dihydrostreptomycin		22
Tetracycline	31.5	24

Table 2. *Mycoplasma laidlawii* agar diffusion bioassay for chloramphenicol, chlortetracycline and tylosin

Chloramphenicol		Chlortetracycline		Tylosin tartrate	
mcg/disc	mm	mcg/disc	mm	mcg/disc	mm
1	16	0.3	7	0.03	12
5	23.5	0.9	15	0.09	15
10	28	3	18	0.3	19.5
20	34	9	22.5	0.9	23.5

These experiments show that *Mycoplasma laidlawii* type B can be used for agar diffusion bioassays much in the same fashion that other microorganisms have been used in the past for antibiotics and antimicrobial substances. In studies with another *Mycoplasma* culture poor results were obtained when the above system was tried; this may have been due to the rather 'special' incubation and nutrition requirements of this culture. The value of the dye reduction technique over visual inspection of growth is quite noticeable since with many *Mycoplasma* growth on agar is not too easy to detect visually and the edge of inhibition zones is often diffuse. However, our bioassay measures only inhibition of respiration (including death) and not growth inhibition, and consequently differs in this additional respect from the assay described by MILLER *et al.*[8].

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