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Antibiotic Activity in the Presence of Agar

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ABSTRACT

Agar has been shown to interfere with the activity of some antibiotics against *Staphylococcus aureus*. This interference has been observed as an increase in the minimal inhibitory concentration and in the diameter of the zone of inhibition. Purifying the agar with water extractions substantially reduced this adverse effect.

Previous studies in this laboratory have shown that the presence of agar in a test medium can seriously interfere with the inhibitory activities of antimicrobial agents (13, 24, 25). In an effort to overcome this interference, agar was extracted with water prior to use in the medium. The washed agar medium was found to be less antagonistic for these inhibitors, and the minimal inhibitory concentration in this medium often approached those in nutrient broth.

It was felt that this antagonistic effect might also extend to the antibiotics. If this were true, it could be an important factor in both the detection and use of these agents. It is well known that in the search for new antibiotic-producing organisms the initial screening tests are routinely performed in an agar medium, and quite often the antibacterial spectrum of a new antibiotic is determined by the agar plate dilution technique (2, 18). In addition, a semisolid medium is routinely used in sensitivity tests to establish the best antibiotic for use in clinical therapy.

This investigation was therefore carried out to determine whether the presence of agar in the test medium had any adverse effect upon the activities of antibiotics, and whether this antagonism could be reduced by the use of washed agar.

MATERIALS AND METHODS

The culture of *Staphylococcus aureus* employed in previous studies (13, 24, 25) was used throughout this investigation. The organisms were stored on nutrient agar at 4 C and were transferred every 4 days. The inoculum was prepared by growing the organisms for 24 hr at 37 C on nutrient agar and washing them from the agar in sterile isotonic saline. A Klett-Summerson photoelectric colorimeter with a blue filter was used to obtain a uniform turbidity of 100 Klett units. After a 10-fold dilution, this suspension contained approximately 2.4×10^7 cells per milliliter.

Concentrated aqueous solutions of the antibiotics were filter-sterilized through an ultrafine Morton fritted-glass filter, and were aseptically diluted to the desired concentration in sterile, distilled water.

The washed agar cubes (3% Difco agar) were prepared in the same manner as described in previous studies (13, 24). The cubes were melted under flowing steam in the autoclave prior to use in the test medium; 3% aqueous solutions of unwashed Difco agar were melted in a similar manner.

Double-strength nutrient broth was prepared, and was divided into three portions. One portion was diluted to single strength with melted, washed agar; the second portion was diluted to single strength with melted, unwashed agar; and the third portion was diluted to single strength with deionized, distilled water. In each of the semisolid media, the final agar concentration was 1.5%.

A 5-ml amount of each medium was pipetted into a series of screw-cap tubes and was autoclaved for 15 min at 121 C. The tubes were then placed in a water bath and were brought to a temperature of 46 C. The tubes were removed individually from the water bath, and 0.1 ml of the antibiotic solution was added aseptically. The tube was then vigorously shaken on a Vortex Jr. mixer. A 0.1-ml amount of the standardized cell suspension was then added, and the tube was again shaken. When the agar had solidified, the tubes were incubated at 37 C for 48 hr. At the end of this period, the tubes were examined under 10× magnification for growth.

The approximate inhibitory concentration for the organism was first determined by a screening test in which concentrations of 0.01, 0.1, 1.0, 10, 100, and 1,000 ppm were used. Once the inhibitory range was determined, it was divided into 10 increments. For example, if the range was 0.1 to 1.0, the increments in this range were 0.1 ppm. Two additional experiments were performed with the use of the smaller increments. All data from these experiments are given in parts per million (ppm) of antibiotic (w/v).

The media for the agar plates were prepared in the same manner as for the tubes. They were autoclaved, cooled to 45 C, and seeded with the standardized suspension of *S. aureus*, and 10 ml was pipetted into sterile petri plates. Antibiotic sensitivity discs (Difco) of intermediate concentration were placed on the

solidified agar, and the plates were refrigerated at 4 C for 2 hr. They were then incubated at 37 C for 48 hr and were examined.

RESULTS

In the control tubes containing no antibiotic, heavy growth occurred at the surface of the agar and slightly below the surface. In subinhibitory concentrations of antibiotics, the organism consistently produced individual colonies on the surface and slightly below.

When the sensitivity of *S. aureus* to the tetracyclines obtained in nutrient broth was compared with that found in nutrient broth solidified with unwashed Difco agar, it was noted that the minimal inhibitory concentrations (MIC) were only slightly higher in the agar medium (Table 1). A comparison of the washed and unwashed agar media reveals that use of the washed agar medium reduced the MIC by one-half. This difference is statistically significant.

The antibiotics shown in Table 2 were not significantly affected by the presence of unwashed or washed agar in the medium.

The activities of the antibiotics presented in Table 3 were markedly affected by the presence of unwashed agar in the test medium. There was a 55-fold increase in the MIC of polymyxin in the semisolid medium, a 30-fold increase for neomycin, a 6-fold increase for kanamycin, and approximately a 3-fold increase for streptomycin. Although the MIC for these compounds was higher in the washed agar medium than in nutrient broth, the major portion of the total antagonism was removed from the agar by washing.

TABLE 1. *Effect of agar on the inhibitory activity of the tetracyclines against Staphylococcus aureus*

Compound	Minimal inhibitory concn (ppm)		
	Nutrient broth	Unwashed agar	Washed agar
Aureomycin hydrochloride	0.15	0.27	0.11
Terramycin hydrochloride	0.46	0.68	0.34
Declomycin hydrochloride	0.07	0.13	0.06

[a] Average of four determinations.

TABLE 2. *Antibiotics that are not significantly affected by the presence of agar*

Compound	Minimal inhibitory concn (ppm)[a]		
	Nutrient broth	Unwashed agar	Washed agar
Vancomycin	0.5	0.7	0.5
Novobiocin sodium	0.9	1.0	0.8
Penicillin G sodium	0.1	0.1	0.1

[a] Average of four determinations.

TABLE 3. *Antibiotics that are adversely affected by the presence of agar*

Compound	Minimal inhibitory concn (ppm)[a]		
	Nutrient broth	Unwashed agar	Washed agar
Polymyxin	4.0	220.0	9.0
Neomycin	0.1	3.0	0.45
Kanamycin sulfate	0.5	3.0	0.6
Streptomycin sulfate	2.25	6.25	4.0

[a] Average of four determinations.

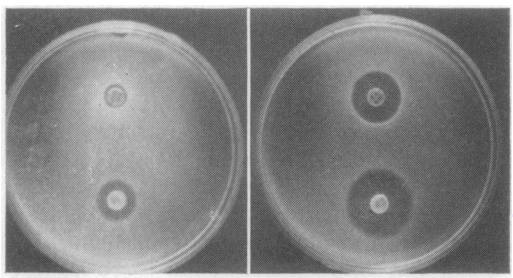

FIG. 1. *Zones of inhibition on unwashed agar medium (left) and washed agar medium (right). Upper disc contains neomycin, lower disc contains dihydrostreptomycin.*

Figure 1 illustrates the effect of washed Difco agar on the zones of inhibition produced by antibiotic discs of neomycin and dihydrostreptomycin. The increase in size of the zone of inhibition produced by neomycin on the washed agar is especially noticeable. Similar experiments with the tetracyclines showed only a slight increase in the size of the zone on washed agar.

DISCUSSION

Our results show that the presence of agar in the test medium may markedly affect the activity of an antibiotic against *S. aureus*. This is to be expected because agar is a chemically reactive substance. According to Araki (1), agar is a complex substance composed of at least two polysaccharides: agarose, which contains little sulfate and no acid groups, and agaropectin, which is sulfated and contains acid residues.

Agar carries an overall negative charge, probably due to the acid and sulfate groups. Because of the presence of these charged groups, agar may

react with protein molecules (3, 32) and basic groups of other molecules such as polymyxin and neomycin (9).

Divalent metallic cations have been shown to reduce the antibiotic effects of polymyxin, neomycin, streptomycin, novobiocin, and particularly the tetracyclines (7, 20, 30, 31, 33). It has been demonstrated that calcium, magnesium, and other metallic cations are often bound to agar (1). Washing the agar indicates that substantial quantities of these ions are removed.

Traces of growth factors and stimulatory factors have also been found in agar, including thiamine, β-alanine, biotin, niacin, and *p*-aminobenzoic acid (6, 21, 22). These factors could alter an organism's sensitivity to an antibiotic in a variety of ways.

This investigation indicates that two important problems are currently being overlooked in many laboratories. First, antibiotics are routinely detected and screened in an agar medium. It is possible that many valuable antibiotics are overlooked or discarded because their effects have been suppressed by the presence of agar. Second, clinical sensitivity tests are usually carried out by use of a medium with agar and sensitivity discs. Eisenberg, Weiss, and Flippin (8) noted that, although 54 strains of *Candida albicans* were sensitive to nystatin in broth, only 11% of these were sensitive to the same concentration of this antibiotic on plates. In only 18.5% of the tests were the results of the plate and tube dilution sensitivity tests in aggreement. Vancomycin, the tetracyclines, streptomycin, nystatin, dihydrostreptomycin, and furadantin have also been shown to be affected by agar (10, 16, 17, 29). In some instances, bacteria that appear resistant to antibiotics by evaluation with sensitivity discs may be rapidly destroyed by the antibiotic in vivo (27).

To eliminate these antibiotic-testing problems, an improved solidifying agent is imperative. The technique of washing agar prior to use has been used in the fields of biochemical genetics (22), immunology (11), and virology (26, 28). This results in the extraction of biologically and chemically active factors (5, 12, 19, 23). Our results indicate that a washed agar medium is superior to an unwashed agar medium for testing antibiotic sensitivities.

Washing alone, however, is not sufficient to make agar an inert component of the test system. Agar still contains acidic groups which are capable of binding positively charged atoms or molecules (4). Several reports (3, 14, 15) indicate that a gel-forming, neutral polysaccharide containing very small quantities of SO_4^- can be obtained from agar. It is quite possible that its reduced chemical reactivity will render it more suitable for use in testing antimicrobial agents.

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