EVALUATION OF ANTISEPTICS BY TEST TUBE METHODS

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Introduction

Many different types of chemicals, including mercurials, dyes, surface tension depressants, and other substances, with water, alcohol, glycerin, and other solvents, are used at present for antiseptic purposes. Considerable variation is noted when various antiseptics are tested by test tube methods using different bacteria. Variation is also noted in results reported by different individuals dealing with the same antiseptic and the same bacterium.

Formerly cultures of typhoid bacilli were extensively used in the laboratory to test antiseptics. In recent years, however, staphylococci, on account of their ubiquity, resistance, and frequency of infection, have largely supplanted the typhoid bacillus as the main test organism. It appears probable that many of the marked differences reported in the antiseptic action of various chemicals against these two species may be attributed to the physical differences of these forms in artificial culture. The typhoid bacillus grows in peptone media rather regularly as an even emulsion, while the staphylococcus grows in grape-like clusters and zoogleal masses which in turn are often clumped in granules of macroscopic size. Lack of more uniform antiseptic action in the laboratory against such physically dissimilar bacteria as typhoid bacilli and staphylococci in artificial culture, therefore, should in a measure be anticipated. The additional factor of chemical selectivity, such as is evidenced in the Gram stain reaction, undoubtedly explains certain other irregular antibacterial properties; however this phase of antiseptic action will not be dealt with here.

As long ago as 1903 Andrews (1) called attention to the fact that mercuric chloride and other mercurials, although used in practical procedures as effective antiseptics, gave unsatisfactory test tube germicidal results with *Staphylococcus aureus* in peptone media. He attributed this lack of complete killing of all bacteria in the culture dose to the zoogleal manner of growth of this organism in ordinary peptone broth cultures. Later Shippen (2) in 1928 called attention to this same phenomenon in tests of mercuric chloride as well as of certain dyes.

Also, it has been shown that some antiseptics which contain surface tension depressants and alcohol as a solvent, while exhibiting considerable antiseptic effect in watery media tests in the laboratory, are almost devoid of antiseptic action when tested against bacteria in a semisolid fibrin medium, thus simulating to some extent the conditions of animal tissues (3). Thus it appears that in many cases rather excellent test tube assays of antiseptics may not be translatable into terms of effectiveness in tissue antisepsis due to the simple fact that semisolid tissues may block the antibacterial mechanism entirely.

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The variable conditions of testing antiseptics against any particular organism which should receive attention include (a) physical condition of the test culture and the relation of this to the medium and temperature of growth, (b) presence of protein, especially semisolid material such as fibrin, (c) temperature of medication, and (d) extent of culturing to determine presence of viable bacteria following medication. The purpose of this paper is to report the action of some commonly used antiseptics upon the staphylococcus under such varied conditions of testing.

EXPERIMENTAL

In the following experiments regular commercial solutions of Merthiolate 1-1000, designated as Antiseptic "A," and three other well known commercial antiseptics designated here as Antiseptic "B" 1-500, Antiseptic "C" 1-1000, and Antiseptic "D" 1-50, were used along with mercuric chloride 1-1000 and various dilutions of phenol for comparison. *Staphylococcus aureus*, Insecticide and Fungicide strain No. 209, was used as the test culture. The stock culture was maintained on beef extract agar slants made with Armour peptone in the regular manner (4). Broth test cultures were prepared in beef extract Armour peptone broth medium, and consisted of the first, second, third, or later, broth cultures in a series originally beginning with the growth on agar.

It may be pointed out that succeeding subcultures of the test organism in Armour peptone broth are frequently increasingly granular in texture and quite unsuitable for biological experimentation (including agglutination, phagocytosis, et cetera), and lead only to confusion if used in chemical antiseptic experiments. Unusual degrees of "resistance" to phenol and other chemicals developed in this way, therefore, includes resistance of masses of cocci which obscures resistance of individual bacteria, and is highly artificial. Low surface tension and "wetting-out" properties of antiseptics promote more rapid killing of such masses of cocci in the test tube. However, these properties are blocked and do not operate in blood clot or protein of the semisolid consistency of tissues.

Table 1 shows a summary of certain comparative germicidal tests selected from a large number conducted during the past year. In these tests *Staphylococcus aureus* culture No. 209 in Armour peptone broth was used and in some instances probably an unusual severity of test conditions has been imposed on the germicides. In these particular tests various degrees of granulation of the test culture were noted, and it was found that if the test culture was quite rough, granular, pellicled, and precipitated, an enhanced resistance to mercurials in aqueous solution at 20° C. resulted, without much change being noted in resistance to chemicals having special wetting-out properties including phenol, alcohol, and surface tension depressant preparations. It may be noticed quite regularly that the test culture No. 209 is unusually granular in Armour peptone broth when grown at 37° C. It has been our experience that this granular characteristic is much less pronounced in similar broths prepared with some other peptones including Parke Davis, Witte, Berna, and Proteose peptones. It appears also that rough culture characteristics, although affecting the outcome of many 20° medication tests, do not have a parallel adverse effect on antiseptic test results of aqueous mercurials when medication is conducted at body temperature.

In conducting the tests recorded in the first column of Table 1 the stock strain of *Staphylococcus aureus* No. 209 was transferred weekly for ten weeks on the usual Armour peptone beef extract agar. A first generation Armour peptone broth culture was then prepared from the last agar culture, incubated twenty-four hours at 37° C., and then subjected to the test, using six germicides, including phenol, as shown.

A dilution of 1-40 phenol killed and 1-50 phenol failed to kill. All of the other five germicides in the dilutions used failed to kill the test culture under these conditions. The experiment shows that an artificial resistance may at times be obtained in a test culture such that both aqueous mercurials and germicides having enhanced wetting-out properties, such as germicide "C" and phenol, fail to kill the entire inoculum of test culture. Usually the first culture generation in broth is not regarded as particularly "resistant."

The test results recorded in columns 2 and 3 in Table 1 show that succeeding broth cultures of staphylococci grown at 37° C. may be quite granular, and would be judged unfit for routine biological tests such as agglutinin and opsonin measurements. When such cultures are medicated for five minutes at 20° C. with mercurials in aqueous solution, incomplete killing may result, while if medicated at 37° C. generally complete killing of the entire inoculum takes place. In such tests using granular culture it may be repeated that both temperature and wetting-out properties of the antiseptic affect the outcome of the tests. Both the microscopic and macroscopic characeristics of such staphylococcal culture are markedly different from those noted in typhoid bacillus cultures, for example, and it appears that in ordinary test methods these more fundamental factors are not generally recognized.

In connection with another series of experiments (using Kendall and other high protein-containing media) we have repeatedly noted the fact that staphylococci grow under such conditions as single forms or diplococci. The clumps seen in the usual laboratory peptone media do not appear here, and attention was directed to a possible bearing which this may have on tissue antisepsis in relation to test tube evaluation methods. It may be said that growth in the high protein media is not so "vigorous" as in peptone broth, and also that such media are "unfavorable" media; however, it would appear that staphylococci in infected tissue in nature have grown in a medium more like the best protein media than artificial peptone media. These are the important organisms in tissue antisepsis. Also it is readily found that the "phenol-resistance" of staphylococcal cultures without the grape-like clumps is only slightly less than that of the usual laboratory cultures, thus corresponding more nearly with other cultures such as typhoid and coli which regularly appear in the laboratory as homogeneous emulsions.

We have tested aqueous mercurials further in a comparative way by test tube methods so modified as to obtain test culture in even emulsions. This may be accomplished in various ways without changing the nature of the medium, one of the simplest of which is allowing the test culture to grow at room temperature instead of at 37° C. By this means the granulation of the culture is greatly delayed. Also if there is logic in conducting antiseptic tests in the laboratory at 20° C., it may be assumed that culture produced under similar conditions should be the

TABLE 1. SUMMARY OF COMPARATIVE GERMICIDE TESTS AGAINST STAPHYLOCOCCUS CULTURE 209

Medication conducted for five minutes followed by plantings into 250 cc. bottles of Armour peptone broth unless otherwise indicated

Germicide	Test temp. 20°C. First generation culture. Grown at 37°C. Very granular.	Test temp. 20°C. Third genration culture. Grown at 37°C. Very granular.	Test temp. 37°C. Third generation culture. Grown at 37°C. Very granular.	Test temp. 20°C. Third generation culture. Grown at 20°C. Smooth and even.	Test temp. 37°C. Fibrin or "white clot" plates.
Merthiolate 1-1000 (Mercurial)	+	±	—	—	—
Germicide B 1-500 (Mercurial)	+	+	—	—	+
Germicide C 1-1000 (Non-mercurial)	+	—	—	—	+
Germicide D 1-50 (Mercurial)	+	+	+	+	+
Mercuric Chloride 1-1000 (Mercurial)	+	+	—	±	+
Phenol 1-40	—	—	—	—	+
50	+	—	—	—	+
60	+	+	—	—	+
70	+	+	—	+	+
80	+	+	+	+	+

All cultures used were Armour peptone broth cultures planted from Armour peptone agar cultures.
Legend: + = growth; — = no growth; ± = irregular.

object of the test. Table 1, column 4, shows results of germicide tests against quite even homogeneous cultures of *Staphylococcus aureus* No. 209 which had been grown at room temperature for twenty-four or forty-eight hours. The tests were otherwise conducted in the usual way, and it appears that the "phenol-resistance" is not much different from that determined with twenty-four hour culture grown at 37° C. The results also show that Merthiolate and germicides "B" and "C" are effective against smooth staphylococcus cultures in vitro, and support the idea that the usual Armour peptone broth cultures grown at 37° C. introduce an artificial factor, i. e., granulation and clumping of masses of bacteria, not present in the original *B. typhosus* tests.

Table 1, column 5, shows the results of further special comparative tests against staphylococci embedded in poured "white clot" (fibrin) agar plates. Each plate contained 0.1 cc. of staphylococcus broth culture and 15 cc. of solid medium. The medium consisted of 50 per cent fresh oxalated plasma and 50 per cent of the usual beef extract agar to which calcium chloride was added, at the same time as the test culture, when the plates were poured. Such plates were exposed to 10 cc. quantities of the various germicides for five minutes, followed by removal of the latter and subsequent incubation of the plates. When Merthiolate was used, uniform sterilization of the plate was accomplished as verified by subculturing, while when other germicides were used growth always resulted. It is certain that tests of this general nature evaluate antibacterial properties of antiseptics (3) more nearly possible of realization in actual tissue antisepsis than the properties indicated by most single test tube methods.

CONCLUSION

Most antiseptic test tube assay methods using staphylococci, when applied to certain aqueous antiseptics such as mercurials, may introduce artificial conditions not present in the older tests using typhoid bacilli. The unique zoogleal and granular manner of growth of the staphylococcus in peptone broth may readily lead to artificial and excessive degrees of mercurial "resistance" not found in smooth cultures nor noted in tests conducted by medicating these bacteria more nearly as in tissue antisepsis.

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