THE ISOLATION OF PROBABLE PATHOGENIC STAPHYLOCOCCI[1]

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In searching for pathogenic types of staphylococci in cultures taken from suspected pathologic sources, it is customary to plate the cultures on suitable media and select colonies for isolation. Tests of pathogenicity are then applied to the pure cultures. Because of the impracticability of testing all colonies, several typical colonies must be selected in the hope that they will include the pathogenic variants. This method has several disadvantages, among which are the following: (a) colonies of pathogenic variants may not be among those selected; (b) much energy must be expended in isolating and testing non-pathogenic types; (c) some of the cultures selected may be duplicates; (d) it is difficult to estimate the number of colonies of pathogenic staphylococci because they cannot be recognized with certainty in the primary cultures; (e) variations in the size and composition of the bacterial cells make it difficult to secure a uniform test dose; (f) for precise work graded amounts of culture must be injected; (g) the results of animal inoculation tests are frequently confused by effects due to emboli, intercurrent infections, etc.; (h) animal inoculation tests are also influenced by the age and state of nutrition of the animals; (i) there is no sharp demarcation between pathogenic and non-pathogenic staphylococci—making it necessary to establish arbitrary standards; and (j) variations in the method of growing and preparing the culture affect the results of animal inoculation tests. Although the pathogenicity of a strain may vary with different species of animals, it is generally conceded

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that, using the term in its broader sense, a strain which is pathogenic for human beings produces dermonecrotic and lethal effects in rabbits.

The medium to be described was developed to simplify search for pathogenic types. On this medium about 98.5 per cent of strains of the pathogenic type of staphylococcus grew luxuriantly, while about 94 per cent of the non-pathogenic type were inhibited. This medium should be useful for isolation purposes, particularly when a large series of cultures is to be tested.

METHODS USED TO ESTIMATE THE ACCURACY OF THE MEDIUM

In order to determine the reliability of the proposed medium, it was necessary to study the pathogenicity of a large number of strains and test their reactions on the medium. Adequate animal inoculation tests of pathogenicity would have involved a tremendous amount of work. The possibility was considered of substituting *in vitro* tests because hemolysis, coagulase and crystal-violet agar tests of staphylococci had been shown to parallel the lethal effects on rabbits (Chapman, Berens, Peters and Curcio, 1934 and Chapman and Berens, 1935). The positive *in vitro* reactions of strains isolated from pus obtained from sinuses, osteomyelitic lesions, boils, carbuncles, etc. and the correlation of the three *in vitro* properties in a large series of strains indicated that hemolysis, coagulase and crystal-violet agar tests could be applied as *in vitro* indicators of probable pathogenicity. The simultaneous use of several *in vitro* tests, each of which correlated with rabbit inoculation tests, increased the accuracy of interpretation of the results.

CALCULATION OF ERRORS OF DIFFERENT TESTS

In order to make appropriate allowance for possible errors an attempt was made to estimate the degree of accuracy of the *in vitro* tests.

Earlier experiments had demonstrated that animal inoculation tests, particularly when only one animal was used, were not as reliable as is often considered. The possible error was calculated

by injecting graded amounts of killed staphylococcal cultures intravenously into 131 rabbits. If a rabbit died within a few minutes after inoculation, or if it died following the injection of a certain amount of culture while other rabbits injected with larger amounts survived, the deaths were considered as errors. On this basis, 21 of the rabbits (16.3 per cent) gave erroneous results. Therefore, the possibility of such errors must be considered in interpreting the results of animal inoculation tests. Obviously, the results will be influenced by the criteria of pathogenicity. No attempt has been made to study this phase of the problem, it being assumed that, with the reservations just discussed, the animal inoculation experiments mentioned in the previous section are acceptable for comparative purposes.

It was shown (Chapman *et al.*, 1934) that some hemolytic albus strains were not pathogenic while some pathogenic aureus strains were not hemolytic. It also appeared that the coagulase test could be used to complement the hemolysis test. When this combination of tests was applied to the study of 119 strains, the results were parallel with those of rabbit inoculation tests in 81.5 per cent of the strains.

In a later paper (Chapman and Berens, 1935), it was shown that strains could be classified according to the color of their growths on 1:300,000 crystal violet agar. The results of this test agreed with those of rabbit inoculation tests in 27 of 28 strains (96.4 per cent).

In some strains in which the results of *in vitro* and *in vivo* tests did not agree, the discrepancy could be explained on the basis of the 16.3 per cent error of single animal inoculation tests.

PARALLEL BETWEEN HEMOLYSIS, COAGULASE AND CRYSTAL-VIOLET AGAR TESTS

In interpreting the results of *in vitro* tests, aureus strains which produced considerable hemolysis on rabbit blood agar were considered as reacting positively to the hemolysis test. Hemolysis tests were not applied to albus strains. Albus and aureus strains that coagulated oxalated human plasma within 12 hours were considered coagulase-positive. Strains which produced violet or

orange growths on crystal-violet agar in 36 hours were considered positive to the crystal-violet agar test.

The results of the crystal-violet agar test were parallel with hemolytic and coagulating properties in 86.4 per cent of 701 aureus strains and 95.7 per cent of 1012 albus strains (table 1). When the results of the three tests did not agree they could be explained on the basis of an intermediate type of culture or by dissociative

TABLE 1

Comparison of hemolysis and coagulase tests with the crystal-violet agar reaction in 1713 strains of staphylococci

TYPE	STRAINS SHOWING	HEMOLYSIS	COAGULASE	CRYSTAL-VIOLET AGAR	NUMBER	PER CENT
Aureus	Agreement	+	+	+	427	
		0	0	0	28	
		0	+	+	148	
		+	0	+	12	
				Total	615	86.4
	Disagreement	+	0	0	3	
		+	+	0	27	
		0	+	0	19	
		0	0	+	37	
				Total	86	13.6
Albus	Agreement		+	+	91	
			0	0	877	
				Total	968	95.7
	Disagreement		+	0	19	
			0	+	25	
				Total	44	4.3

influences. Even in "pure" cultures of pathogenic strains non-pathogenic variants may appear soon after isolation.

CONSTANCY OF THE IN VITRO REACTIONS

The stability of the reactions was tested by applying the three *in vitro* tests both at the time of isolation and again after storage in the refrigerator for one month. Significant differences were noted in 14 of 200 hemolysis tests (7 per cent), in 12 of 191

crystal-violet agar tests (6.3 per cent) and in 6 of 173 coagulase tests (3.4 per cent).

THE VALUE OF IN VITRO TESTS

When allowances were made for possible errors of the different tests, the agreement with animal inoculation tests and the close agreement between the three *in vitro* tests indicated that they could be used for the study of large numbers of cultures where extensive animal inoculation tests would involve an enormous amount of work.

With due regard to the limitations just discussed, the *in vitro* tests were applied as tests of probable pathogenicity, subject to confirmation by rabbit inoculation tests.

THE DEVELOPMENT OF A MEDIUM FOR THE ISOLATION OF PROBABLE PATHOGENIC STAPHYLOCOCCI

Although the results of crystal-violet agar tests were parallel with those of rabbit inoculation and hemolysis and coagulase tests, it was necessary to inoculate heavily to secure growth on crystal-violet agar. For this reason, it is not suitable for the isolation of staphylococci.

Search for a medium containing a dye which would differentiate pathogenic from non-pathogenic strains, and yet not inhibit pathogenic strains, led to discovery of the value of bromthymol-blue agar. Although the early results with solid media containing bromthymol blue were shown to be due to the indicator property of the dye (Chapman, 1936) it was found that, when the concentration of dye was increased, non-pathogenic staphylococci were inhibited. Best results were obtained with a concentration of 0.17 gram per liter. To determine whether this concentration of bromthymol blue had an inhibitive effect on pathogenic types of staphylococci, swabs from the nose, throat, gum margins, etc. of patients suspected of having chronic diseases were plated on proteose lactose agar containing 0.017 per cent bromthymol blue. Duplicate swabs were plated on rabbit blood agar. The number of colonies on bromthymol-blue agar was estimated and compared with the number of *in vitro* positive colonies isolated from rabbit-blood agar. Parallel results were obtained in 69 of 96 pairs of

swabs and widely different results were obtained in only 3 pairs. Except for a few intermediate size colonies of *in vitro* negative strains, those strains which grew produced colonies as large as those on ordinary media.

TABLE 2

Probable errors of crystal-violet agar and bromthymol-blue agar (pH 6.8) tests

Total number of strains examined........................ 240

Strains showing agreement between bromthymol-blue agar and crystal-violet agar tests........................ 222 (92.5%)

STRAIN NUMBER	TYPE	DISTRIBUTION OF THE 18 STRAINS SHOWING DISAGREEMENT					
		Hemolysis	Coagulase	Crystal-violet agar	Growth on bromthymol-blue agar	Probable error of	
						Crystal-violet agar test	Bromthymol-blue agar test
3782	Aureus	0	0	+	0	+	
3860	Aureus	+	+	0	+	+	
3892	Aureus	+	+	0	+	+	
3893	Aureus	0	+	0	+	+	
3910	Aureus	0	+	0	+	+	
4067	Albus		+	0	+	+	
4076	Albus		0	+	0	+	
4078	Albus		0	+	0	+	
3737	Aureus	+	+	0	0	+	+
3878	Aureus	0	+	0	0	+	+
3915	Aureus	+	+	0	0	+	+
3917	Aureus	0	+	0	0	+	+
3916	Aureus	+	+	0	0	+	+
3966	Aureus	0	0	+	+	+	+
3961	Albus		+	0	0	+	+
3864	Aureus	+	+	+	0		+
4058	Albus		0	0	+		+
4065	Albus		+	+	0		+
Total errors						15 (6.4%)	10 (4.3%)

GROWTH OF OTHER BACTERIA ON BROMTHYMOL-BLUE AGAR

In addition to pathogenic staphylococci, other bacteria, particularly members of the colon-aerogenes group, also grew well on bromthymol-blue agar and could not be eliminated by bacteriostatic methods. However, the appearance of colonies of

pathogenic staphylococci is so characteristic that they cannot be confused.

COMPARISON OF ABILITY TO GROW ON BROMTHYMOL-BLUE AGAR WITH THE POWER TO PRODUCE ORANGE OR VIOLET GROWTHS ON CRYSTAL-VIOLET AGAR

In tests of pure cultures, the results of crystal-violet agar and bromthymol-blue agar tests were parallel in 92.5 per cent of 240 strains (table 2). The bromthymol-blue agar test was considered correct in 95.7 per cent of the strains. The majority of errors were due to *in vitro* positive strains that failed to grow, and attempts were made to eliminate this fault.

EFFECT OF CHANGING THE HYDROGEN-ION CONCENTRATION OF BROMTHYMOL-BLUE AGAR

Tests were made to determine whether changes in the hydrogen-ion concentration of bromthymol-blue agar would affect the growth of staphylococci. Various batches of bromthymol-blue agar were prepared and the pH adjusted to 7.0, 8.0, 9.0 and 10.0 before sterilizing. More acid media were too inhibitive for staphylococci. A series of 105 strains was plated on these media and on stock bromthymol-blue agar (pH 6.8 after sterilization). Best results were obtained with the medium which had been adjusted to about pH 10.0 with thymol-blue indicator. The reaction of this medium after sterilization was about pH 8.6. Parallel results were obtained in 98 strains. In 5 instances, *in vitro* positive strains grew better than on stock bromthymol-blue agar. Only one *in vitro* positive and one *in vitro* doubtful strain grew better on the stock medium. It was decided to adjust the medium to about pH 9.6 with the hydrogen electrode before sterilization. After sterilization the reaction was about pH 8.6 with the hydrogen electrode.

COMPARISON OF IN VITRO TESTS OF STAPHYLOCOCCI WITH THE POWER OF THE CULTURES TO GROW ON ALKALINE BROMTHYMOL-BLUE AGAR

A series of 276 strains was plated on this alkaline medium, with the results listed in table 3. The hemolysis test was con-

sidered erroneous in 7.2 per cent of the strains, the crystal-violet agar test in 2.9 per cent, and the coagulase test in 1.1 per cent. The bromthymol-blue agar test gave erroneous results in 7.7 per

TABLE 3

Comparison of different in vitro tests of staphylococci

STRAINS SHOWING	TYPE	HEMOLYSIS	COAGULASE	CRYSTAL-VIOLET AGAR	ALKALINE BROMTHYMOL-BLUE AGAR	NUMBER OF STRAINS	PER CENT OF TOTAL STRAINS
Agreement in all tests	Aureus	+	+	+	+		
	Albus		+	+	+	219	79.3
	Albus and aureus	0	0	0	0		
Probable error of the hemolysis test	Aureus	0	+	+	+	19	7.2
	Aureus	+	0	0	0	1	
Probable error of crystal-violet agar test	Aureus	+	+	0	+	1	2.9
	Aureus	0	0	+	0	3	
	Albus		0	+	0	3	
	Albus		+	0	+	1	
Probable error of bromthymol-blue agar test	Albus and aureus	0	0	0	+	17	6.2
	Aureus	+	+	+	0	1	1.5
	Albus		+	+	0	3	
Probable error of coagulase test	Aureus	+	0	+	+	2	1.1
	Albus		+	0	0	1	
Unclassified	Albus		0	+	+	1	1.8
	Aureus	0	+	+	0	1	
	Aureus	0	0	+	+	3	
Total						276	

cent of the strains. In 17 of the latter instances (6.2 per cent) the error was due to growth of *in vitro* negative strains while, in 4 strains (1.5 per cent), it was due to the failure of *in vitro* positive strains to grow. If the strains growing on bromthymol-blue agar

could be confirmed by other *in vitro* tests, the error of the bromthymol-blue agar method of isolation should be reduced to about 1.5 per cent.

Actual tests of crude cultures indicated that those *in vitro* positive strains that failed to grow in mass culture were not entirely lost because there were a few cells in those cultures which were capable of growing on bromthymol-blue agar.

RESULTS OBTAINED BY PLATING THE ORIGINAL (CRUDE) CULTURES ON BROMTHYMOL-BLUE AGAR AND CONFIRMING THE GROWTHS BY CRYSTAL-VIOLET AGAR AND BY COAGULASE TESTS

Tests were made by plating 81 pairs of swabs from the nose, throat, etc. on bromthymol-blue agar, which had the following composition:

Beef extract	3 grams
Proteose peptone, Difco	5 grams
Lactose	10 grams
Agar	15 grams
Bromthymol blue	0.17 gram
Water to make	1000 cc.

The reaction is adjusted before sterilization to about pH 9.6 with the hydrogen electrode.

In 36 hours, staphylococcal colonies are raised, smooth, entire and opaque. They are similar in morphology to the colonies on ordinary media. The size varies from 1 to 1.5 mm. in diameter depending upon the distribution, colonies on crowded plates being smaller than well-isolated colonies. About 90 per cent are deep yellow while about 10 per cent are gray with a blue tinge. The color does not seem to be significant.

One swab was rubbed first on bromthymol-blue agar and then on rabbit-blood agar. A second swab from the same source was rubbed first on rabbit-blood agar and then on bromthymol-blue agar. This reduced errors due to disproportionate sampling. The inoculum was spread by means of glass spreaders and incubated for 36 hours.

Growths from bromthymol-blue agar were confirmed by (a) transplanting them to crystal-violet agar and incubating for 36

hours and (b) mixing 1 loopful of the growth with 0.5 cc. of oxalated human plasma and incubating overnight. The growths from rabbit-blood agar were transferred to nutrient agar for purification of the different types and *in vitro* tests were applied to the pure cultures. The number of *in vitro* positive colonies was the

TABLE 4
Probable accuracy of different tests of staphylococci

TEST	COMPARISON WITH	NUMBER OF TESTS	PROBABLE ACCURACY
			per cent
Rabbit inoculation	Different amounts of culture	132 rabbits	83.7
Hemolysis	Other *in vitro* tests	276 strains	92.8
	Stability of reaction after storage	200 strains	93.0
Coagulase	Other *in vitro* tests	276 strains	98.9
	Stability of reaction after storage	173 strains	96.6
Hemolysis-coagulase combination	Rabbit inoculation	119 rabbits	81.5
	Crystal-violet agar	1012 albus strains	95.7
		701 aureus strains	86.4
Crystal-violet agar	Rabbit inoculation	28 rabbits	96.4
	Hemolysis-coagulase combination	1012 albus strains	95.7
		701 aureus strains	86.4
	Other *in vitro* tests	240 strains	93.6
	Stability of reaction after storage	191 strains	93.7
Bromthymol-blue agar	Direct isolation (unconfirmed)	96 pairs of swabs	96.9
	Direct isolation (confirmed)	81 pairs of swabs	98.8
	Other *in vitro* tests*	276 strains	92.3

* Most of the error was due to the growth of *in vitro* negative strains. When the growths are confirmed by other *in vitro* tests, the apparent error is reduced to 1.5 per cent, or less.

same by both methods in 71 of the 81 pairs of swabs. In 5 swabs, more *in vitro* positive colonies were recovered by the bromthymol-blue agar method while, in 5 swabs, more *in vitro* positive colonies were recovered by the rabbit blood agar method. Except for one pair of swabs giving an occasional *in vitro* positive colony

on bromthymol-blue agar but a moderate number of *in vitro* positive colonies on rabbit-blood agar the results were essentially similar in both series of swabs.

The deep yellow color carried over from the growths on bromthymol-blue agar sometimes obscured the color of the growths on crystal-violet agar. This was taken into consideration when the results of the crystal-violet agar and coagulase confirmation tests did not agree.

In table 4 an attempt has been made to evaluate the results of different tests. From these results it would appear that, when properly interpreted, hemolysis, coagulase, crystal-violet agar and bromthymol-blue agar tests of staphylococci are sufficiently reliable to be used as indicators of probable pathogenicity.

When the bromthymol-blue agar method of isolation was applied to the study of about 200 crude cultures, 73 of 86 growths on bromthymol-blue agar were considered *in vitro* positive, 7 did not give parallel results to all tests and were considered intermediate in pathogenicity, while 6 growths were considered *in vitro* negative. The proportion of *in vitro* negative strains in this series was similar to that obtained in previous series of tests.

CONCLUSIONS

The power to grow on 0.017 per cent bromthymol-blue lactose agar pH 8.6 is a useful characteristic for the differentiation of staphylococci. It may be used for the isolation of probable pathogenic staphylococci in cases where rabbit inoculation tests are impractical.

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