SODIUM CHLORIDE MEDIA FOR THE SEPARATION OF CERTAIN GRAM-POSITIVE COCCI FROM GRAM-NEGATIVE BACILLI

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In culturing mixed clinical specimens, such as urines, in which there are both Gram-positive cocci and Gram-negative bacilli, the difficulty of obtaining pure cultures of the cocci by the usual methods of plating is at times insurmountable. This is especially so when the cocci are present in relatively small numbers, or when the bacilli belong in such genera as Proteus or Pseudomonas. The recent work of one of the authors (White, 1929) showed that colon group organisms could be inhibited or killed by concentrations of urea or of sodium chloride which failed to destroy *Staphylococcus aureus*. This finding suggested the possibility of using such substances in media for the culturing of specimens from cases of mixed infection.

Study of the effect of sodium chloride upon bacteria has been undertaken by many workers. Martens (1888) found that staphylococci were viable on transfer from 30 per cent solution. Petterson (1900), Guillermard (1908, 1909), and others have observed the selective action of salt. So far as we can determine, however, the application of such data for the isolation of salt resistant organisms does not seem to have been considered before. Fischer (1903) classified bacteria in two groups, according to the permeability of their membranes. Among those with permeable membranes, that is, which could not be plasmolyzed, he placed, among others, the Gram-positive spore-formers studied, Proteus, *Escherichia acidilactici*, the sarcinae and the staphylococci. The organisms with impermeable membranes, that is, capable of

undergoing plasmolysis, included the spirilla, *Eberthella typhi*, *Escherichia coli-communis*, *Pseudomonas pyocyanea* and others. Lewandowsky (1904) believed that the action of high percentages of salt upon bacteria was due to the molecular concentrations of the solutions. Holzinger (1908) has shown the inhibitory action of osmosis upon bacteria. The bactericidal action of physiological salt solutions has been demonstrated especially by Duthoit (1923a, 1923b), who found that *Staphylococcus aureus* was the most resistant to physiological salt solution of the organisms studied. In a third paper Duthoit (1923c) showed the retardation of this bactericidal action of sodium chloride by the addition of calcium chloride. Schmidt (1924) transferring to liquid instead of solid media, was unable to confirm Duthoit's results, a difference which may possibly be explained by this variation in method. Neither Duthoit nor Schmidt has considered the reaction of the test solutions.

This report consists of the study of 50 cultures on media containing sodium chloride in concentrations from 1 through 25 per cent. Tests have been made with pure cultures, with cultures mixed in different proportions in the laboratory, and with clinical specimens which showed microscopically the presence of two or more types of organisms.

The following media have been used:

1. Sodium chloride agars. These were beef infusion agars, containing 500 grams of ground beef per liter of water, 1.5 per cent agar, 1 per cent peptone and from 1 through 20 per cent sodium chloride. This medium was adjusted to pH 6.0, tubed, autoclaved and slanted.

2. Sodium chloride broths. These were beef infusion broths, containing 500 grams of ground beef per liter of water, 1 per cent peptone and from 1 through 25 per cent sodium chloride. These broths were adjusted to pH 6.0, placed in flasks in 200 cc. amounts and autoclaved. For the tests, 1 cc. amounts were transferred to small sterile tubes, sterility controls being made at 37.5°C.

Tubes which stood more than twenty-four hours before use were plugged with sterile rubber stoppers, in order to prevent

evaporation, while flasks were kept with their cotton plugs wrapped in tightly fastened rubber.

The hydrogen ion concentration was invariably 6.0 because this had been found by White (1929) to be about the average hydrogen concentration of dog urines in which differential bacteriostasis was first obtained. It is possible that further work will result in the use of a different hydrogen ion concentration, as other combinations of the variables are studied.

The following cultures have been studied:

	Number of strains	
Staphylococcus aureus	5	(nos. 1, 2, 3, 209, 12)
Staphylococcus albus	4	(nos. 5, 6, 8, 11)
Micrococci	5	(nos. 4, 7, 9, 10, 13)
Eberthella typhi	1	
Salmonella paratyphi	1	
Salmonella schottmulleri	1	
Eberthella paradysenteriae Flexner	1	
Eberthella dysenteriae Shiga	1	
Pseudomonas aeruginosa (*B. pyocyaneus*)	5	
Proteus vulgaris Hauser	4	
Klebsiella pneumoniae (*B. pneumoniae*)	1	
Colon group bacilli	18	
genus Escherichia	10	(nos. 28, 32, 127, 153, 1, 9, 38, 75, 92, 138)
genus Aerobacter	8	(nos. 25, 89, 170, 73, 90, 96, 190, 8)
Bacillus anthracis	1	
Corynebacterium pseudodiphtheriticum	2	

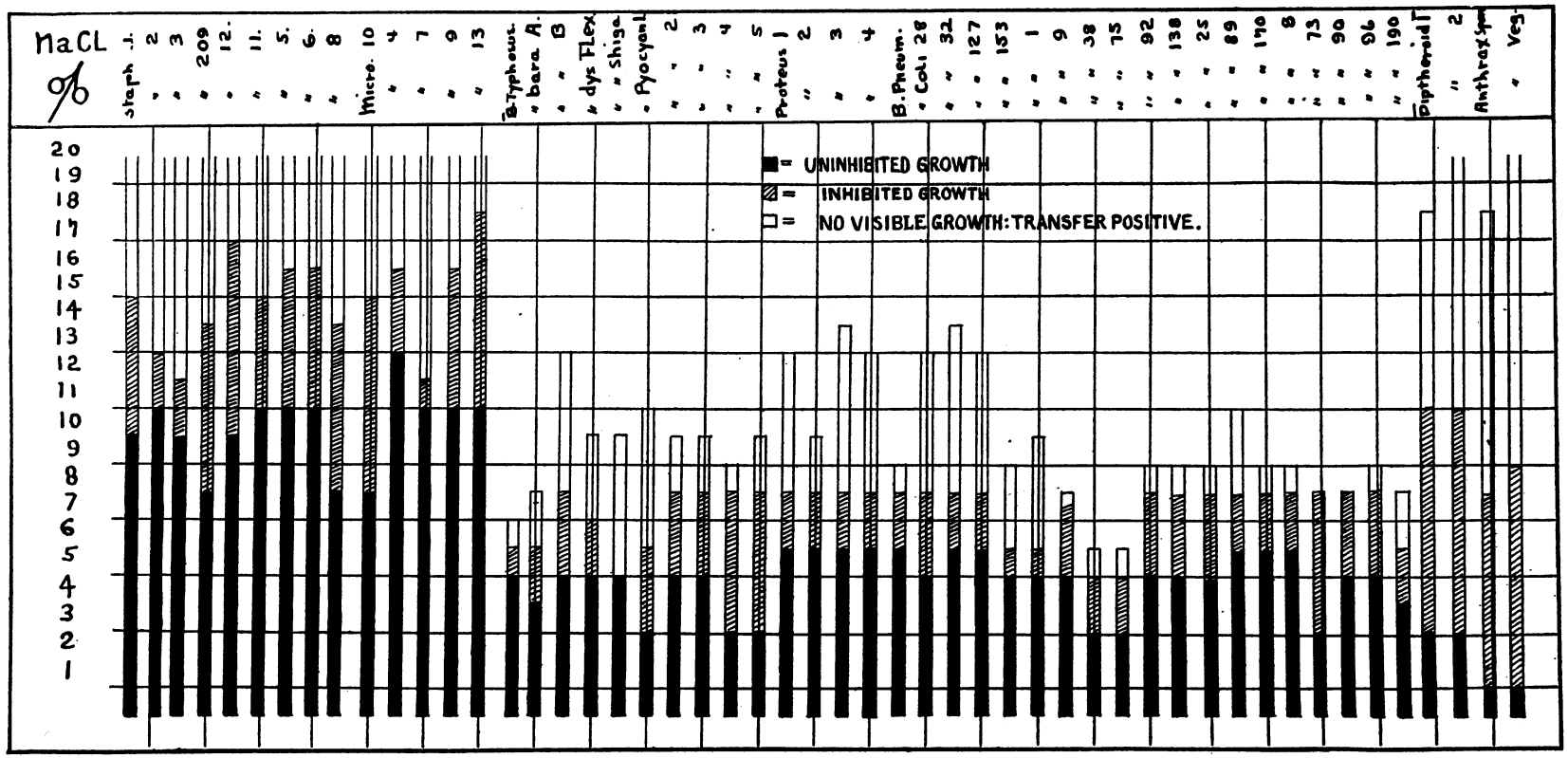
TESTS WITH PURE CULTURES

1. Salt agars

Series of slants, prepared as described, and containing from 1 through 20 per cent sodium chloride, were inoculated with 1 standard loopful (5 mm. oise) of an eighteen-hour pH 7.6 broth culture of the test organism. The effect of the acidity of the medium was controlled by comparison with the growth on a similarly inoculated slant of 1 per cent salt, pH 7.6 agar. The effect of the salt concentration was controlled by the 1 per cent salt pH 6.0 slant. Readings were made after twenty-four and forty-eight hours of incubation at 37.5°C. At the end of this

time, transfers were made to 10 cc. of pH 7.6 broth from slants which showed no visible growth, the surface of the slants being carefully scraped. These transfer tubes were incubated for forty-eight hours. We were therefore able to determine three or four zones of action; first, the salt concentrations which gave no visible inhibition of growth; second, the zone of inhibited but definite growth; third, the zone in which there was no visible growth, but in which there was inhibition without complete killing, as evidenced by positive transfers; and fourth, the zone of salt concentrations in which there was actual killing of the organisms, the transfers being sterile. The results of these tests are recorded in bar diagram I. In this, the solid black bars represent the zones of uninhibited growth, the stripped bars the zones of visible but inhibited growth, and the outlined bars the zones of no visible growth from which positive transfers were obtained. In some cases the bars are not closed, indicating that transfers from the highest salt concentration studied, 20 per cent, were positive. A vertical line closing the outlined bar indicates that transfers from greater concentrations were sterile.

It will be seen from this diagram that there is a sharp differentiation between the cocci and both the Gram-negative and the Gram-positive bacilli. All of the 14 cultures of cocci studied grew heavily through 8 per cent salt, while none of the Gram-negative bacilli grew heavily on higher than 6 per cent salt, some showing inhibition in as low as 4 per cent. Moreover, 3 strains of the cocci grew heavily on 10 per cent salt; 7, or 50 per cent of the strains showed no inhibition on 11 per cent; and 1 strain was uninhibited on 13 per cent. That is, the break between uninhibited and inhibited growth of the cocci lay at about 11 per cent salt, while with the Gram-negative bacilli it came at 5 or 6 per cent. Similarly, the zone of visible but inhibited growth of the cocci lay between 9 and 19 per cent salt as compared with 4 through 8 per cent for the Gram-negative bacilli. The third zone, that of complete inhibition of visible growth, without killing, as shown by positive transfers, began with the cocci at 12 per cent salt. Its upper range was not determined for these organisms, as all of the cocci grew on transfer from 20 per cent



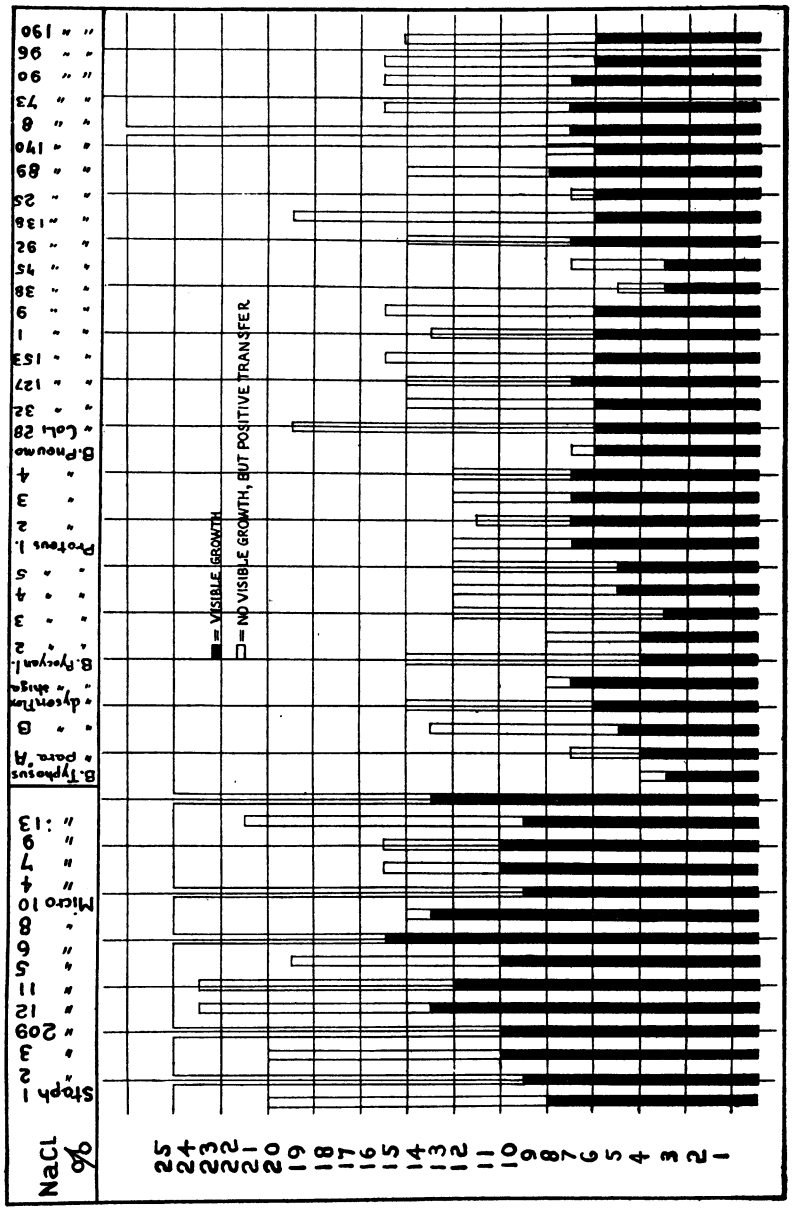
BAR DIAGRAM I. GROWTH OF BACTERIA ON SALT AGARS

salt. With the Gram-negative bacilli, however, this third zone began at 6 per cent and in no case extended beyond 14, all transfers from greater concentrations being sterile. This seems to demonstrate clearly the differential bacteriostasis of sodium chloride, to which the cocci are more resistant than the bacilli.

In regard to the Gram-positive bacilli, it is of interest to note that their growth is inhibited more easily than any of the other organisms studied, the two diphtheroids growing heavily only through 3 per cent salt, the vegetative and sporulating anthrax culture showing inhibition above 1 per cent salt. However, the zone of visible but inhibited growth with all of the cultures of Gram-positive bacilli equals or slightly exceeds the similar zone for the Gram-negative bacilli. The third zone, with the Gram-positive bacilli, is more comparable to that of the cocci, although the vegetative anthrax culture and 1 of the diphtheroids were killed by 19 per cent salt. These results seem to indicate an intermediate position for the Gram-positive bacilli, but it is impossible to draw further conclusions in regard to these organisms until a large number of strains have been studied. Koch, 1881, was the first to show the resistance of anthrax spores to salt, while both de Freytag, 1890, and Stadler, 1899, confirming Koch's statement in regard to spores, found that the vegetative forms of *B. anthracis* were not resistant to salt. By de Freytag's, 1890, method diphtheria bacilli were not killed in three weeks by salt, Stadtler's, 1899, transfers of this organism being positive after four and one-half weeks of salting. Schmidt, 1924, however, found that this organism was soon killed by different salt concentrations in comparison with his results with other organisms. None of these authors, however, so far as can be determined, noted the reaction of their media.

2. Salt broths

One cubic centimeter of broth, prepared as described, was placed in a small tube and inoculated with 1 standard loopful of an eighteen-hour pH 7.6 broth culture of the test organism. Salt concentrations from 1 through 25 per cent were used. The effect of the acidity of the medium was controlled by comparison



BAR DIAGRAM II. GROWTH OF BACTERIA IN SALT BATHS

with growth in pH 7.6, 1 per cent salt broth, and the effect of the salt by comparison with the 1 per cent, pH 6.0 broth. After twenty-four hours' incubation at 37.5°C., readings were made to determine the presence or absence of visible growth. One-tenth cubic centimeter was then transferred from each tube to 10 cc. of pH 7.6 broth. The transfer tubes were incubated for forty-eight hours at 37.5°C. By these tests it was possible to determine; first, the zone of salt concentration which allowed visible growth; second, the zone of inhibition of growth without complete killing, as shown by positive transfers; and third, in some cases, the zone of complete killing, as shown by sterile transfers. The results of these tests are expressed in bar diagram II.

It is evident that the selective action of sodium chloride may be demonstrated in liquid media, although they are more favorable to the growth of the rapidly developing bacilli. All of the cocci grew well in 9 per cent salt broth, most of them in higher concentrations, 1 strain even in 16 per cent. No visible growth of the bacilli was observed, however, in more than 9 per cent salt. That is, the maximum for the bacilli was the minimum for the cocci. The zone of inhibition of growth without killing showed less striking comparisons between the cocci and bacilli in broth than on agar, but the difference was, in general, still demonstrable. Eighteen cultures, or 53 per cent of the Gram-negative bacilli were killed by the salt concentration of 15 per cent, which was tolerated by the most susceptible of the cocci. The average salt concentration tolerated by the cocci, as shown by positive transfers, was at least 21.7 per cent, while for the Gram-negative bacilli it was 13.6 per cent.

Certain observations may be made in regard to the salt tolerances of the organisms studied. *Eberthella typhi* was the least resistant of all of our cultures. Although de Freytag, 1890, and Stadtler, 1899, by their methods of salting well-developed cultures, obtained positive transfers after long periods of time, other authors, using methods more comparable to ours, have obtained results similar to ours. Thus, Matzuschita, 1900, found the growth of *Eberthella typhi* good on from 0 to 3.5 per cent salt, moderate from 4.5 to 5.5 per cent, scarce on 6.5 per cent and

slight or none above this. K. von Karaffa-Korbutt, 1912, found that this organism grew in 7 per cent salt in peptone broth, but not in 8 per cent. Although Schmidt (1924) found *Eberthella typhi* viable in 1.5 per cent salt after ten days, Duthoit (1923a), reported that in 0.9 per cent salt two thousand bacilli were reduced to six within six and one-half hours. It is possible that a study of a number of strains of this organism and of the related forms will reveal some specific differences.

The effect of salt upon organisms of the genera Pseudomonas and Proteus was striking. Of the 5 cultures of *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*) studied, 3 grew heavily on agar only through 3 per cent salt, the other 2 cultures being inhibited above 5 per cent. These 5 cultures were killed on salt agar by a concentration of not more than 11 per cent salt, as compared with growth on transfer of all the cocci from 20 per cent. The four Proteus strains behaved alike on salt agar, that is, they all grew heavily on 6 per cent salt, with inhibition through 8 per cent, no transfer being positive above 14 per cent. The findings with both of these genera on salt broth showed a general parallelism, with a somewhat greater tolerance. The repression of chromogenesis in the Pseudomonas cultures was invariable. Matzuchita, 1900, also found Proteus more resistant to salt than Pseudomonas, growth of the former being poor above 8.5 per cent, the latter, scarce at 6.5 per cent.

In analyzing the findings with organisms of the colon group and its related form, *Klebsiella pneumoniae*, our lack of adequate classification makes it impossible to draw accurate comparisons. There are no marked differences between cultures of the genus Escherichia and those of the genus Aerobacter. These organisms, regardless of their genera, have been studied by India ink examinations for the presence of capsules, in fact some of them were selected from a collection of 200 cultures on account of their encapsulation. Of the 19 organisms, including the Friedländer bacillus 9, or 47.3 per cent were heavily encapsulated and viscid in growth, 10, or 52.6 per cent showed no capsules, or very slight ones and were not viscid in growth. Of the 14 cultures which were killed by 9 per cent agar, 9, or 64.2 per cent were the 9

thickly encapsulated cultures. That is, all of the thickly encapsulated cultures belonged in the group most easily killed by salt.

TESTS WITH MIXED CULTURES

The preceding experiments indicated the possibility of using salt agars to inhibit bacilli present in mixed cultures, or even, in some instances, to isolate Gram-positive cocci from such mixtures of organisms. Eighteen-hour pH 7.6 broth cultures of the test organisms were mixed in two proportions. Series A consisted of 1 cc. of the culture of a bacillus and 1 cc. of the culture of a coccus, while Series B consisted of 1 cc. of the bacillus culture and 1 standard loopful of the coccus culture. One loopful of such mixtures was placed on each of the following agar slants; 1 per cent salt, pH 6.0 agar, the control and on 6, 8, 10 and 15 per cent salt, pH 6.0 agars. After forty-eight hours of incubation, smears of the slants showing visible growth were examined. Transfers were made to 10 cc. of pH 7.6 broth from the 10 and 15 per cent salt agars, whether or not there was any visible growth. The use of a liquid medium was favorable to the development of any bacilli which might be viable.

Enrichment of the cocci was obtained on all of the salt agars tested. The controls on 1 per cent salt, pH 7.6 agar, on both series, had heavy growth, either of both organisms, or of an overwhelming number of bacilli. In the A series of salt agar tests, in which the inoculum contained equal parts of the bacillus and of the coccus cultures, examinations of smears of the cultures showed only cocci in 75 per cent of the tests on 6 per cent salt; 71.4 per cent on 8 per cent salt; 89.2 per cent on 10 per cent salt and 92.8 per cent on 15 per cent salt. Transfers in this series to pH 7.6 broth gave pure cultures of the cocci in 17.8 per cent from 10 per cent salt and in 39.2 per cent from 15 per cent salt. In the B series of experiments, in which the inoculum was 1 loopful of a mixture of 1 cc. of the bacillus culture and 1 loopful of the coccus culture, the results on 8, 10 and 15 per cent salt were fully as good, in some cases better, than in series A. On 6 per cent salt, in series B, 67.6 per cent of the smears showed only

cocci; on 8 per cent salt, the number of smears showing only cocci increased to 91.1 per cent. On 10 per cent salt, 94.1 per cent of the series B tests showed only cocci, but only 11.6 per cent of the transfers gave pure cultures of cocci. On 15 per cent salt all of the tests in series B which had any growth, showed only cocci, while of the 27 viable transfers from this concentration, 16 or 59.2 per cent were pure cultures of cocci.

It is of interest to note that the amount of growth on salt agar seems to be determined by the number of cocci in the inoculum. In the B series, in which there were fewer cocci, the growth was generally lighter, although the number of bacilli was relatively larger than in the A series. This finding correlates with the results of examinations of smears of the cultures.

TESTS WITH MIXED INFECTIONS

In the application of the previous findings for the enrichment of cocci in mixed infections, the salt concentrations employed must be of sufficiently wide range to cover the unknown variations in the number of organisms present. In general results with lower concentrations of salt may be obtained from clinical material than from pure or artificially mixed cultures. This is probably on account of the smaller number of organisms present.

In table 1 are summarized the findings from 20 cases of mixed infection.

The organisms found in these infections were staphylococci, streptococci of the Gamma type, colon group bacilli and *Pseudomonos pyocyanea*, from 2 to 4 types of organisms being present. In 16 of these 20 cases, pure cultures of the cocci were obtained from 6 through 15 per cent salt agar. Of the 4 cases in which there was enrichment of the cocci, but in which pure cultures were not obtained, 3 were not placed on high salt concentrations. In the fourth case, no. 20, a streptococcus was present in very small numbers with *Ps. pyocyanea* and a colon group bacillus. It seems evident from these 20 cases that the use of salt agars is of definite advantage in culturing many specimens from mixed infections.

TABLE 1

Summary of findings of salt agar cultures of 20 cases of mixed infection

CASE NUMBER	SOURCE	ORGANISMS PRESENT IN DIRECT SMEAR	ORGANISMS OBTAINED BY ROUTINE CULTURE	SALT AGAR CULTURES		
				Salt used	Salt giving mixed cultures	Salt giving pure coccus
				per cent	*per cent*	*per cent*
1	Urine	Many bacilli; few cocci	Colon group bacillus; *Staph. albus*	6, 7, 8, 9, 10	7	8 (9 and 10 sterile)
2	Urine	Same as case 1	Same as case 1	6, 7, 8, 9, 10	7	8, 9, 10
3	Wound	Same as case 1	Same as case 1	7, 8, 9	None	7, 8, 9
4	Wound	Same as case 1	Colon group bacillus; *Staph. aureus*	6, 7, 8	6, 7	8
5	Wound	Same as case 1	Colon group bacillus; *Staph. albus*	6, 8, 10, 15	6, 8, 10	15
6	Wound	Same as case 1	Colon group bacillus; *Staph. albus*	5, 6, 7, 8	5, 6, 7, 8	None
7	Urine	Small number of bacilli and cocci	*B. pyocyaneus; Staph. albus*	6, 8, 10, 15	6, 8	10, 15
8	Urine	Small number of bacilli; very few cocci	Colon group bacillus; *B. pyocyaneus; Staph. albus*	6, 7, 8	6, 7	8
9	Urine	Same as case 8	Same as case 8	6, 7, 8	6, 7	8
10	Urine	Many bacilli and cocci	Colon group bacillus; diphtheroid; *Staph. albus*	6, 7, 8, 9, 10, 15	6, 7, 8, 9	10, 15
11	Wound	Same as case 10	Same as case 10	5, 6, 7	5, 6, 7	None
12	Wound	Same as case 10	Same as case 10	6, 7, 8	None	6, 7 (8 sterile)
13	Wound	Same as case 10	Same as case 10	6, 7	6	7
14	Wound	Same as case 10	Same as case 10	6, 7, 8	6, 7, 8	None
15	Wound	Same as case 10	Same as case 10	6, 7, 8	6, 7	8
16	Wound	Same as case 10	Same as case 10	6, 7, 8	6, 7	8

17	Wound	Same as case 10	Colon group bacillus; *B. pyocyaneus;* diphtheroid; *Staph. albus*	6, 7, 8	6, 7	8
18	Urine	Small number of bacilli; many streptococci	Colon group bacillus; Streptococcus (Gamma)	6, 7, 8	None	6, 7, 8 (plain and blood salt agars)
19	Abscess	Many bacilli and streptococci	Colon group bacillus; Streptococcus (Gamma)	6, 7, 8	None	6, 7 (8 sterile)
20	Urine	Many bacilli; few streptococci	Colon group bacillus; *B. pyocyaneus;* Streptococcus (Gamma)	6, 8, 10, 15	6, 8, 10, 15	None

DISCUSSION

These experiments demonstrate some of the possibilities of the use of salt agars. Should the method prove of value, it may be applied to other organisms than those included here. A study of the use of different salt concentrations in special media for streptococci is indicated from the few cases we have observed. While there is little evidence that salt offers any aid to generic or specific identification, although this was once claimed by Dubois, 1910, for *Escherichia coli*, it is possible that further studies will reveal such differences. We hope that others will test this method or some modification of it on similar or on different types of specimens.

SUMMARY AND CONCLUSIONS

1. It has been found that pH 6.0 sodium chloride agars, with salt concentrations from 2 through 20 per cent, exert marked inhibitory action on the growth of bacilli of the typhoid, paratyphoid, dysentery, and colon groups, on species of Proteus, Pseudomonas, on diphtheroids and on *Bacillus anthracis*. The Gram-positive cocci studied tolerate high salt concentrations, all being positive on transfer from 20 per cent sodium chloride agar.

2. In pH 6.0 broths, with salt concentrations from 2 through 25 per cent, the same differential bacteriostasis may be observed, although to a lesser degree than on agar.

3. It has been found that when mixtures of cocci and bacilli in different proportions are cultured on appropriate salt agars, the cocci invariably outgrow the bacilli and may sometimes be recovered in pure culture.

4. The use of 6, 8, 10 and 15 per cent salt agars greatly facilitates the isolation of Gram-positive cocci from specimens from mixed infections.

5. The use of such salt agars is therefore suggested for the inhibition of Gram-negative bacilli and for the isolation of Gram-positive cocci.

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