THE COMPARATIVE VIABILITY OF PNEUMOCOCCI ON SOLID AND ON FLUID CULTURE MEDIA.*

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It has long been recognized among bacteriologists that the viability of the pneumococcus on artificial culture media varies. Kruse[1] asks why it is that the pneumococcus will sometimes refuse to grow upon a medium prepared in the standard manner with especial regard to the proper reaction. Experience in this laboratory has shown that no difficulty is found in obtaining abundant growths of pneumococci in broth prepared as will be described later, provided that enough infected material is introduced, but that sometimes the necessary quantity is very large, especially if large volumes of broth are inoculated. It has also been observed that the amount necessary to produce a growth in broth may be larger than that necessary to produce a growth on agar. We have found that a loop of infected blood is generally sufficient to set up a growth in five cubic centimeters of broth, but if this be inoculated into half a liter of broth, no growth occurs. In order to obtain a growth in a half liter or liter flask, it is usually necessary to inoculate with five cubic centimeters of young cultures. A series of studies was undertaken to obtain more accurate data in regard to the observed facts and, if possible, to shed some light on the essential nature of these phenomena. The following paper forms a brief report of these studies.

METHODS.

The broth used for all experiments was a beef infusion containing 0.5 per cent. of sodium chloride and 1 per cent. of Witte's peptone (Kahlbaum), sterilized intermittently in streaming steam for short periods. The reaction was 0.6 per cent. acid to phenolphthalein. The agar used in all experiments contained 0.5 per cent.

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[1] Kruse, W., Allgemeine Mikrobiologie, Leipzig, 1910, 158.

glucose. Sheep serum glucose agar contained 20 per cent. of sheep serum sterilized separately at a low temperature. All the broth employed in each experiment, including that used for dilutions, was of a single lot. In making inoculations into broth, the material was introduced carefully in the middle of the surface, care being taken not to infect the sides of the tube.

DIFFERENCE OF VIABILITY OF THE PNEUMOCOCCUS WHEN INOCULATED INTO BROTH AND ON AGAR.

The method employed was to inoculate varying amounts of pneumococcus culture into broth and on sheep serum glucose agar, to determine the amount necessary in each case for growth to occur. Thirty-three cultures were so tested. The strains tested included representatives of groups I and II, as described by Dochez,[2] as well as *Streptococcus* or *Pneumococcus mucosus*. The cultures were obtained directly from the heart's blood of inoculated animals. All the strains tested were of high virulence, not more than 0.000,001 of a cubic centimeter of the broth culture being required to kill a mouse. In each case one loop of heart's blood from the mouse was inoculated into five cubic centimeters of bouillon. After twenty-four hours' growth, progressive dilutions were made from this culture as follows: A series of tubes was taken, each tube containing five cubic centimeters of bouillon. To the first was added 0.5 of a cubic centimeter of the culture and a mixture made by rotating the tube, care being taken to produce as little foam as possible. This is called dilution tube 1. Of this mixture, 0.5 of a cubic centimeter was taken and added to the second tube containing five cubic centimeters of bouillon and mixed as before. This was called dilution tube 2. In a similar manner successive dilutions were made, in each case 0.5 of cubic centimeter of the mixture being added to the succeeding tube to form the next lower dilution. Now from each one of these dilution tubes, one drop was added to a tube containing five cubic centimeters of bouillon, and a mixture made as before, and one drop was added to a tube of slanted agar and distributed over the surface. A similar inoculation of one drop from the undiluted culture was also made in broth and on agar. These latter

[2] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

inoculations are numbered 0 in the protocols. The tubes were then observed daily for at least five days to determine whether or not growth had occurred. If growth occurred on the agar, it was evident in twenty-four hours. In the broth, if growth occurred at all, it was usually heavy in twenty-four to forty-eight hours, but in a few instances the growth was apparent only after seventy-two hours. Whenever it occurred, it was abundant. In no case was there observed only a slight initial growth without further development. The following table shows a typical result obtained in one of these experiments.

TABLE I.

Pneumococcus I.

	0	1	2	3	4	5	6	7	8
Dilutions	−	+	+	−	−	−	−	−	−
5 c.c. broth	+	−	−	−	−	−	−	−	−
Slant agar	+	+	+	+	+	+	+	−	−

From this experiment it is apparent that many more organisms were required to start the broth cultures than were necessary to produce a growth on agar. By calculation it was determined that about 1,000,000 organisms viable on agar were required to produce a growth in five cubic centimeters of bouillon. Similar results were obtained with all the strains tested, though the results were not quite so striking in every instance. In four of the experiments the results were somewhat discordant, growth being obtained in certain of the higher dilutions when not occurring in the lower.

It has been found convenient, in estimating the comparative viability of pneumococci in broth and on agar, to speak of a broth agar difference, meaning by this the difference in the numbers of tubes in which growth occurs, when the technique as described above is employed. For instance, when growth occurs in broth only in tube 1, while on agar growth occurs in all the tubes up to and including No. 8, the broth agar difference is spoken of as seven tubes. From such differences have been calculated the approximate numbers of cocci viable on agar necessary to start a growth in broth. Table II shows the results obtained in the thirty-three experiments so expressed.

TABLE II.

Determinations by Means of the Broth Agar Difference of the Numbers of Pneumococci Viable on Agar Which Are Necessary to Start a Growth in Broth.

Broth agar difference, in tubes.	Number of cocci viable on agar required to start a growth in broth.	Number of strains tested.
7	10,000,000	1
6	1,000,000	7
5	100,000	6
4	10,000	3
3	1,000	6
2	100	3
1	10	2
0	1	4
−1	0.1	1

EFFECT OF REPEATED PASSAGE ON ARTIFICIAL MEDIUM.

It was soon found that the broth agar difference became less and even finally could be made to disappear, if, instead of employing cultures directly from the animal body, growths from broth after repeated and continuous passage through this medium were used. In one experiment, for instance, the broth agar difference of a culture obtained directly from the animal body was five tubes. After two transfers through broth the broth agar difference was only four tubes; after five transfers, the difference was only three tubes; after fifteen transfers only two tubes; and after seventeen transfers the broth agar difference had entirely disappeared. In other cases so large a number of transfers was not required to bring about this change. Repeated reinoculation on agar had essentially the same effect.

EXPERIMENTS UNDERTAKEN TO EXPLAIN THE BROTH AGAR DIFFERENCE.

The possibility at once suggested itself that the reason why growth from a small number of organisms is more likely to occur on agar than in broth is that the agar medium is better suited to growth of pneumococci and contains substances which are favorable to its growth. Comparative tests showed no differences between glucose agar and sheep serum glucose agar. It seemed possible, however, to test this hypothesis by retaining only the broth as a culture medium and still obtaining an arrangement that would

reproduce to a large extent the solid surface of the agar. This was done as follows: Narrow strips of Schleicher and Schuell's No. 597 filter paper were taken and folded twice, so that when placed in test-tubes a flat shelf would be formed, supported by two legs resting on the bottom of the test-tube. A number of such strips were prepared and placed in test-tubes and sterilized. Then into the tubes broth was carefully poured, so as to wet all parts of the paper and to fill the tubes to within about two centimeters of the transverse shelf. Inoculations were then made into these tubes by placing a drop of the diluted culture on the filter paper shelf, instead of mixing it with the bouillon, as was done in the tubes containing no filter paper. A large number of experiments were performed to compare the broth agar difference as determined in the usual way with the broth filter paper difference. Table III gives the results obtained in this series of experiments. All the cultures employed were obtained directly from the animal body, as in the first experiments described.

TABLE III.

No. of experiment	1	2	3	4	5	6	7	8	9	10	11	12
Broth agar difference, in tubes.	5	6	2	6	2	2	2	1	0	—1	—1	—1
Broth filter paper difference..	5	6	2	6	2	2	2	1	0	—1	—1	—1

It is evident that when the growth on filter paper in broth is compared with the growth in broth alone, the same differences, though to slightly less extent, are seen as are found when growths on agar and in bouillon are compared.

In such experiments the imitation of a solid medium by the filter paper expedient is not perfect. Not all the inoculated material remains on the paper and, on the other hand, in some instances growth may not extend to the broth below and so the occurrence of growth may be overlooked. These experiments indicate clearly that chemical differences alone between the two sorts of medium cannot be responsible for the phenomenon under consideration.

It was next suggested that the difference might be due to difference in available oxygen, the supply of oxygen being much better at the surface of a solid medium than in the depths of the fluid. To test this hypothesis inoculations were made into tubes of melted

glucose agar, which were then allowed to harden, as well as on the surface of agar tubes. The results obtained were compared with the results obtained by making corresponding inoculations into bouillon. In this case the organisms in the depths of the solid medium were under at least as unfavorable conditions as regards oxygen supply as were the organisms in the fluid medium. No essential differences were found between the growths when the inoculations were made into the depths of solid medium from those when they were made on the surface; in both cases growth occurred from inoculation with equally small numbers of organisms and from inoculation with much smaller numbers than were necessary to produce growth in broth. Comparative tests were also made between growths in broth in the ordinary way and broth incubated in an atmosphere of oxygen. The oxygen had no apparent influence in inducing growth to occur from higher dilutions. It therefore seems that the slight difference in oxygen supply on the surface of solid medium and in the depths of liquid medium is not sufficient to explain the broth agar difference.

DISCUSSION.

From the experiments described it would seem that neither chemical differences in composition of culture medium nor differences in available oxygen are sufficient to explain why in one case on solid medium a few bacteria are able to live and multiply and, in the other case, in liquid medium they fail to multiply and die.

A possible explanation is that the organisms in some way assist each other in their growth when they are closely approximated, and when they become scattered in fluid medium this favoring action is absent. It is possible that in their metabolic processes something is produced which is favorable to further multiplication, and in fluid medium this is diffused and diluted. Some years ago certain writers concluded from experiments on viability and on rate of reproduction, that microörganisms sometimes produce substances which favor their multiplication. Wildiers[3] apparently was the first to make this inference. His studies were made on yeasts. Among others who made similar inferences should be mentioned

[3] Wildiers, E., *La cellule,* 1901, xviii, 313.

Rahn,[4] who found that the time required for cell division, as determined for *Bacillus fluorescens liquefaciens* by plate counts, decreased for a while after growth started, to rise later as the culture grew old. The criticism generally made, however, against such conclusions[5] is that traces of poisons (copper and the like) may be present in ordinary culture media, and that these may exert a sufficient effect on very small numbers of bacteria to produce the phenomenon noted.

A second possibility is that such phenomena occur only during adjustment to a new environment, and that local irregularities of diffusion play a part. It may be conceived that when large numbers of organisms are introduced into fluid medium only a few happen to receive (through diffusion) a sufficient supply of nutrient material without experiencing too abrupt a change of environment.

CONCLUSIONS.

1. Pneumococci, when freshly isolated from the body, are able to live and multiply when a small number of them are inoculated into a small amount of broth. If, however, the inoculations are made in large amounts of broth, many more bacteria must be inoculated in order that they may grow.

2. It requires much smaller numbers of pneumococci to start a growth on agar than are required to start a growth in broth.

3. This predilection for solid medium disappears when the bacteria are grown for some time outside the body.

4. This phenomenon is not dependent on differences in chemical composition between the two media employed or on the presence of more available oxygen in one case than in the other.

5. It is probably dependent entirely on physical differences in the two kinds of media, and bears some relation to the differences in possibilities for diffusion in the two media.

[4] Rahn, O., *Centralbl. f. Bakteriol., 2te Abt.*, 1906, xvi, 417, 609.
[5] Kruse, W., *loc. cit.*, p. 167.