A NEW CULTURE MEDIUM FOR THE ISOLATION OF BACILLUS TYPHOSUS FROM STOOLS*

PLATE 20

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Of the numerous methods which have been recommended for the isolation of typhoid bacilli from feces the one at present most widely used is that devised by Endo in 1903. It allows the development of a high percentage of the typhoid organisms inoculated on it, but does not inhibit to any practical extent the growth of those fecal bacteria which develop on ordinary nutrient agar. The great value of the method lies in its sharp differentiation between the lactose-fermenting organisms and those organisms which do not ferment lactose. The colonies of the lactose-fermenting organisms are colored red after 24 hours' incubation, while the other colonies remain colorless. We have found that better results are obtained if both lactose and saccharose are added to the Endo medium, since certain members of the colon-bacillus group ferment saccharose more rapidly than lactose and hence their colonies take on the red color sooner and can no longer be regarded as slow colonies; the typhoid colony is without color in 24 hours, as on the Endo medium containing lactose alone.

The chief disadvantage of the Endo medium lies in the fact that the red color is not confined to colonies of B. coli themselves, but spreads out through the medium adjacent to them. If the colonies of B. coli are close together the whole plate soon becomes red and then colorless colonies on it can no longer be distinguished from the red ones. We have tested a great number of stains separately and in combinations of varying strengths with the view of overcoming this difficulty while at the same time preserving the effectiveness of the medium for the sure growth of typhoid. In every instance where the acid or the basic fuchsin was tried, the color diffused into the medium around the red colonies, so that it was difficult, if not impossible, to recognize the typhoid colonies which may have lain in these areas. We have finally devised a medium that gives even better dif-

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ferentiation between the lactose-fermenting colonies and those that do not ferment lactose than the Endo plate and at the same time remains unchanged in the areas between the colonies. It consists of a combination of methylene blue and eosin in nutrient agar containing lactose and saccharose. On plates of this medium after 18 hours' incubation the colonies of typhoid are colorless and transparent, while those of B. coli are a deep black and do not transmit light. The medium immediately around the colonies of B. coli remains practically unchanged. Hence the plate is workable even when the colonies are close together. More of the feces, therefore, can be safely inoculated on this medium than on the Endo plate and it is to that extent more delicate and to be preferred.

The medium is prepared as follows: Nutrient agar is made in the usual way, containing 1.5% agar, 1% Witte's peptone, 0.5% sodium chlorid, and 0.5% Liebig's meat extract, to the liter of distilled water. It is cleared with egg-white, placed in flasks, and sterilized in the Arnold sterilizer on 3 successive days. The reaction is brought to +0.8. The agar is melted and saccharose (.5%) and lactose (.5%) are added. The medium is then heated for 10 minutes in the Arnold. To every 50 c.c. of the medium are added 1 c.c. of 2% yellowish eosin and 1 c.c. of 0.5% methylene blue. We always add the eosin first and then the methylene blue. The mixture is shaken and plates are poured. The surface of the medium is dried in the usual way before the plates are inoculated. We have also obtained excellent results by substituting for Liebig's extract, meat infusion rendered free from sugar by incubation with B. coli.

Stock solutions of 2% eosin and 0.5% methylene blue in distilled water are kept in the dark. We have not sterilized these solutions, as we found that they could be kept in the ice-box for weeks without causing contaminations of the medium. Ordinarily we do not heat the agar after the dyes are added, but we have demonstrated that the stained agar can be heated a half hour in the Arnold sterilizer without injury.

Eosin and methylene blue in distilled water in the proportion used, give precipitation; in the medium the agar acts as a "Schutzkolloid," preventing the formation of a precipitate.

The following experiment shows that methylene blue in even twice the amount contained in the medium as described, whether alone or in combination with eosin, does not inhibit the growth of the typhoid

bacillus. A strain of B. typhosus freshly isolated from the blood of a patient by culturing in bile was used. Two suspensions of the bacilli were prepared in salt solution and 1 loop of each suspension was inoculated on half of a plate, as shown in Table 1. The colonies were counted after 24 hours' incubation. They were of practically the same size on all the plates. The same nutrient agar with a reaction of + 0.7 was used throughout.

TABLE 1

EXPERIMENT SHOWING THAT TYPHOID BACILLI ARE NOT INHIBITED BY METHYLENE BLUE

Medium Used	Number of Colonies	
	Typhoid Suspension 1	Typhoid Suspension 2
Plain nutrient agar	139	25
Agar + methylene blue (0.02%)	155	21
Agar + eosin (0.04%) + methylene blue (0.02%)	135	26
Agar + eosin (0.04%)	125	20

The following experiment was carried out to determine whether there is any inhibition of the typhoid bacillus when a typhoid stool is inoculated on the plate. A portion of the typhoid stool was rubbed up in salt solution and filtered first through a thin layer of absorbent cotton and then through filter paper. This filtrate was shaken and dilutions of 1 : 10 and 1 : 100 were prepared in salt solution. After this treatment it may be assumed that the typhoid bacilli present are distributed uniformly in the suspensions of the feces. One loop of each of the dilutions was inoculated on an Endo plate, on our methylene-blue eosin plate, and on a plain agar plate. The series of plates containing a convenient number of colonies was worked up in each instance with the results shown in Table 2.

Table 2 shows conclusively that typhoid bacilli in stools develop as readily on the methylene-blue eosin plate as on Endo plates or plain agar.

The chief advantage of this medium over the Endo plate, as already mentioned, consists in the fact that the colonies of B. coli become sharply differentiated from the typhoid bacilli without affecting the medium lying between the colonies, so that a typhoid colony can be readily recognized tho lying in close proximity to numbers of colonies of B. coli. There is of course a sharp limit to the amount of feces that can be safely inoculated on this medium, as is true of

every other medium. If the plate contains tens of thousands of colonies, each individual colony of B. coli remains very small and is poorly, if at all, differentiated from the typhoid colonies. It is certain, however, that this plate will stand a heavier inoculation than the Endo or the Conradi Drigalski plates.

The differentiation between the colonies of B. coli and those of the organisms that fail to ferment lactose is evident by reflected, as well as by transmitted, light; in selecting the suspicious colonies for fishing we use both, but rely chiefly on the picture afforded by the transmitted light.

TABLE 2

EXPERIMENT SHOWING THAT TYPHOID BACILLI INOCULATED FROM STOOLS ARE NOT INHIBITED BY METHYLENE BLUE

Patient	Medium	Total Number of Colonies	Number of Typhoid Colonies
J. L.	Endo	31	1
	Methylene-blue, eosin	42	3
	Plain agar	38	3
M. M.	Endo	29	15
	Methylene-blue, eosin	28	16
	Plain agar	37	22
M. O.	Endo	11	4
	Methylene-blue, eosin	12	4
	Plain agar	4	2
M. B.	Endo	44	21
	Methylene-blue, eosin	35	20

In addition to this main advantage the methylene-blue eosin plate possesses the following minor advantages:

1. The colonies of B. coli are differentiated earlier on this plate than on the Endo plate; that is, if the two plates are inoculated at the same time, the colonies of B. coli on the methylene-blue eosin plate in some instances will have taken on black centers while those on the Endo plate are still colorless or have merely a pink tinge.

2. A greater percentage of the colorless colonies turn out to be typhoid on the methylene-blue eosin plate than on the Endo plate. This is probably due in part to the fact that the former medium contains both lactose and saccharose, while the latter contains lactose alone, and in part to the fact that some of the organisms producing colorless colonies on the Endo plate fail to grow on the methylene-blue eosin plate.

3. There is complete inhibition of certain organisms which form small colonies on the Endo plates.

4. Certain bacteria which give colorless colonies on the Endo medium yield colonies with blue centers and transparent peripheries on our plate.

5. The Endo plate gradually turns pink on exposure to light; our plate remains unchanged. We have left it exposed to diffuse daylight for a period of 3 hours before inoculation without causing any noticeable deterioration.

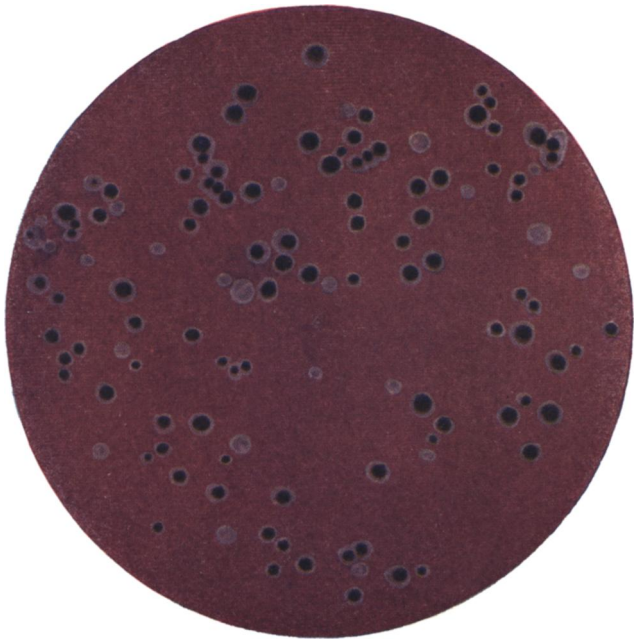
6. The Endo agar is adjusted to + 0.2, a reaction too alkaline to permit of the optimal growth of the typhoid bacillus. Our medium yields good results in agar, the reaction of which is best suited for typhoid. Slight variations in the reaction of the nutrient agar from which it is made, do not affect the efficacy of the methylene-blue eosin medium.

7. Since the medium around the colonies remains unchanged anyway, there is no reason for making the agar stiffer than it is in ordinary use.

Experiments were made also with the eosin methylene-blue combination of stains in nutrient agar with the addition of lactose, saccharose, and dextrose, and with the addition of dextrose alone, with a view to differentiating between typhoid and other non-lactose-fermenting colonies. There is no marked difference between the result given by the 3 sugars from that given by dextrose alone. On a plate containing the stains with 0.25% dextrose in agar, typhoid colonies grow well and are unmistakably differentiated from other colorless colonies when examined under the low power of the microscope, for they have peculiar blue centers which look like fine matted blue hairs. However, as this picture was not constant when typhoid stools were used, we decided to abandon the use of dextrose and depend on the colorless transparent colony on the eosin methylene-blue plate containing saccharose and lactose for diagnosis. If this plate be incubated for 48 hours, the typhoid colonies, even here, will often assume the centers described.

We have used this medium for the examination of scores of typhoid stools and mixtures of normal stools and typhoid cultures with very satisfactory results.

PLATE 20



Reproduction of a methylene-blue eosin plate inoculated with a typhoid stool and incubated for 18 hours. The colonies of B. coli are black. The typhoid colonies are transparent. The largest transparent colonies are not typhoid.