A SELECTIVE MEDIUM FOR ORAL FUSOBACTERIA

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There have been several reports on the use of special selective media for the isolation of oral fusobacteria. Slanetz and Rettger (1933) used a potato extract agar medium containing gentian violet as a bacteriostatic agent. This medium and various modifications of it have been employed by many other investigators. Spaulding and Rettger (1937*b*) and Bøe (1941) used ascitic fluid, blood serum and various vegetable extracts as enrichments to improve the growth promoting qualities of the potato extract medium. In most instances gentian violet, crystal violet, or brilliant green was added to the medium at concentrations of 1:2,000 to 1:20,000 for selective bacteriostasis. For example, Spaulding and Rettger (1937*b*) found that the addition of gentian violet at a concentration of 1:20,000 in potato extract-cysteine agar gave the most effective medium for the isolation of oral fusobacteria. The fusobacteria have greater resistance to these dyes than many other microorganisms, but inhibition of growth of associated organisms was not always accomplished.

In a previous study (Omata, 1951) in this laboratory, in which the inhibitory action of various antibiotics was tested on fusiform bacilli, it was found that certain strains of fusobacteria showed marked resistance to streptomycin and dihydrostreptomycin. In another study (Omata, 1953) it was found that casein digest and yeast extract were essential for excellent growth of oral fusobacteria. A new medium was formulated incorporating streptomycin and crystal violet, and compared with the potato extract-gentian violet medium as to its effectiveness for the isolation of oral fusobacteria from stimulated saliva from humans. This report describes this medium and the results of this comparison.

MATERIALS AND METHODS

The composition of the basal medium (described as FM medium) employed in the study is as follows: Casitone (Difco), 1.5 per cent; yeast extract (Difco), 0.5 per cent; glucose (anhydrous), 0.5 per cent; NaCl, 0.5 per cent; L-cystine, 0.075 per cent; crystal violet, 0.001 per cent; streptomycin, 0.001 per cent; agar, 1.5 per cent and the pH adjusted to pH 7.2. It was derived from fluid thioglycollate medium (Difco Manual, 1953). The sodium thioglycollate has been omitted since it was found in preliminary studies that this compound partially inhibited the growth of pure cultures of fusobacteria.

Contrary to the reports of Spaulding and Rettger (1937*b*) and Bøe (1941) that L-cysteine hydrochloride was essential for the growth of fusobacteria in potato extract agar, it was found in this study that L-cysteine hydrochloride, when added to the basal medium, was somewhat inhibitory for the fusobacteria from pure cultures and from saliva samples.

The FM medium, without streptomycin, was dispensed in 20-ml amounts in 25- by 150-mm test tubes and autoclaved at 120 C for 15 min. It was enriched by adding either 0.5 per cent corn starch (Argo), 5 per cent sterile human ascitic fluid, or 5 per cent sterile horse serum. The starch, when used, was incorporated directly into the basal medium upon preparation. The ascitic fluid and horse serum were added to sterile petri plates just prior to pouring. The streptomycin solution (1:1,000 dilution) was sterilized by filtration and 0.2 ml added to the plates at time of pouring.

The potato extract medium used for comparison was prepared as described by Slanetz and Rettger (1933) and Spaulding and Rettger (1937*a*).

One hundred and two samples of paraffin stimulated salivas were collected from 99 human subjects, generally from five individuals at a time. The samples were shaken manually; a portion was taken from each sample and diluted 1:1,000 and 1:10,000 with sterile dilution broth of the following composition: casitone (Difco), 1.5 per cent; yeast extract (Difco), 0.5 per cent;

NaCl, 0.25 per cent; and L-cystine, 0.075 per cent (pH 7.2). One-ml amounts of the diluted saliva samples were cultured as pour plates in each of the four media tested.

After inoculation the plates were placed in anaerobic jars, which were then evacuated with an aspirating water pump and refilled with a gas mixture of 5 per cent carbon dioxide and 95 per cent nitrogen. This process was repeated three times. The final gas pressure was adjusted to approximately 350 mm Hg. The jars were incubated at 37 C for 72 hr.

After incubation each culture plate was examined with the aid of a stereomicroscope and the colonies counted. Isolations from representative colonies were inoculated into the sterile dilution broth, which was modified with the addition of 0.5 per cent glucose. After inoculation a strip of sterile lead acetate paper was suspended in each tube. The cultures were incubated under the same anaerobic conditions as previously described. After 48 hr incubation the lead acetate strips were observed for hydrogen sulfide production and the cultures tested for indole production with Kovac's reagent (Conn, 1951). Smears were stained by Gram's method for microscopic examination. The isolates of fusobacteria were differentiated on the basis of hydrogen sulfide and indole production, colonial appearance in primary plates, and cellular morphology.

RESULTS AND DISCUSSION

The number of fusobacteria per ml of saliva obtained with the various media are given in table 1. Comparison of the mean counts shows that each of the three experimental media was much more effective in promoting the growth of

TABLE 1

Comparison of mean counts of fusobacteria isolated from stimulated human salivas on specified media

Medium	Mean Count per ml × 10³	No. of Negative Plates
Basal + starch	563	7*
Basal + ascitic fluid	854	6*
Basal + horse serum	836	2*
Potato extract agar	26	42†

* From total of 101 plates.

† From total of 99 plates.

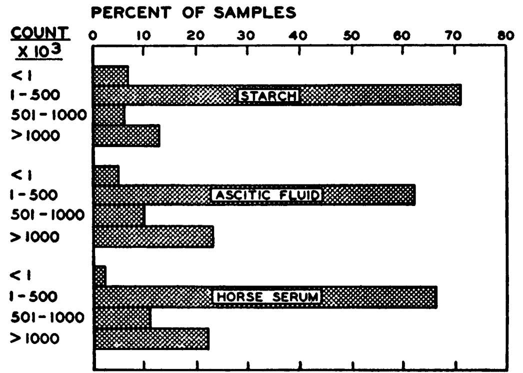

Figure 1. Frequency distribution of fusobacteria counts on experimental media.

fusobacteria than the classical potato extract agar. The basal medium supplemented with ascitic fluid or horse serum supported approximately equal numbers of colonies and yielded counts 50 per cent greater than those obtained on the medium containing corn starch. The medium containing ascitic fluid yielded the highest counts; colonies of fusobacteria were also larger on this medium than on any of the other three. In a recent report, Kasai (1955) has shown that the addition of soluble starch and blood derivatives were beneficial for the growth of filamentous oral bacteria.

The incidence of negative plates was of the same order of magnitude for each of the three experimental media, whereas the potato extract medium yielded at least six times as many negative plates. Composite information of the fusobacteria counts on the three media is given in figure 1. It can be seen that the frequency distributions are very similar.

Four hundred isolations of fusobacteria were made from the various media. These strains have been classified according to two biochemical reactions, namely indole and hydrogen sulfide production (table 2). Biochemical type I corresponds to group I of Spaulding and Rettger, while type II corresponds to group II of Spaulding and Rettger (1937*a*). Isolates listed under type III include strains exhibiting biochemical reactions intermediate between those of types I and II in that these strains either produced indole and not hydrogen sulfide or vice versa.

Strains listed as type I are morphologically similar to *Fusobacterium nucleatum* strain Knorr, with cells 0.5 by 4 to 6 μ in size (Breed *et al.*,

TABLE 2
Biochemical types of fusobacteria isolated from specified media

Medium	No. of Isolates	Type I*		Type II†		Type III‡	
		No.	%	No.	%	No.	%
Basal + starch	113	36	31.8	49	43.4	28	24.8
Basal + ascitic fluid	147	49	33.3	54	36.7	44	30.0
Basal + horse serum	140	51	36.4	52	37.2	37	26.4

* Type I: produced both H_2S and indole.
† Type II: did not produce H_2S or indole.
‡ Type III: variable H_2S and indole production.

1948). All strains produce both hydrogen sulfide and indole; the subsurface colonies are iridescent, wedge-shaped with fine veinlike surface markings, and are very friable.

Type II strains appeared to be identical morphologically with *Fusobacterium plauti-vincenti* strain Knorr (Breed *et al.*, 1948). They do not produce indole nor hydrogen sulfide. Subsurface colonies are iridescent, lobate, spherical and finely veined. The surface colonies are tough and hard to break up.

Most of the isolates listed under type III are consistent with the morphology of *Fusobacterium polymorphum* strain Knorr (Breed *et al.*, 1948), which form cells 0.5 by 8 to 10 μ, and form iridescent, finely veined, lens-shaped colonies in deep agar. However, a few strains morphologically similar to *F. nucleatum* were listed under type III because of their intermediate biochemical reactions. The fourth species, *Fusobacterium biacutum*, was not encountered.

Previous investigators have used gentian violet in concentrations from 1:5,000 to 1:20,000 in various media. In this study, the use of 1:20,000 crystal violet in potato extract medium was found to be unsatisfactory in that a high percentage of the colonies that appeared were contaminants, usually gram negative cocci and rods, and also, fewer fusobacteria colonies appeared. It was found in preliminary experiments that concentrations of crystal violet as low as 1:50,000 in the basal medium were somewhat inhibitory for fusobacteria.

A previous study in this laboratory (Omata, 1951) showed that certain strains of fusobacteria were resistant to the inhibitory action of streptomycin. It was found that the combination of crystal violet and streptomycin in the concentrations used in the FM medium in 1:100,000 dilutions, was effective in suppressing the growth of concomitant bacteria, while not significantly affecting the growth of fusobacteria. For example, with the use of these agents only about 5 per cent of the isolations made were found to be microorganisms other than fusobacteria, the most frequent contaminant being gram negative cocci. A gram negative rod and a yeast were found to be contaminants once each.

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SUMMARY

A selective medium for the isolation and enumeration of oral fusobacteria has been described. The selective agents used are crystal violet and streptomycin, at 1:100,000 dilutions, which effectively inhibit the growth of most concomitant bacteria in human saliva.

Using any one of these three enriched media, strains representing three major species of the genus *Fusobacterium* (*F. nucleatum*, *F. plauti-vincenti*, and *F. polymorphum*) were isolated from human saliva.

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