A NEW ENRICHMENT MEDIUM FOR CERTAIN SALMONELLAE

BY

F. RAPPAPORT, N. KONFORTI, AND BETTY NAVON

From the Municipal Hadassa Hospital, Tel-Aviv, Israel

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A great number of enrichment media for *Salmonellae* have been described in the medical literature. Some of them have gained wide acceptance in many clinical laboratories. Two of the most frequently used enrichment media are tetrathionate broth in various modifications (Muller, 1923; Kauffmann, 1930; Preuss, 1949; Knox, Gell, and Pollock, 1942) and selenite broth (Hobbs and Allison, 1945).

The purpose of an enrichment medium for *Salmonellae* is twofold: it has to enable the pathogens to multiply freely and it should inhibit or kill all accompanying coliform organisms. Particularly troublesome are *Proteus*, *Pseudomonas pyocyanea* and *Aerobacter aerogenes*, which are not always inhibited in the enrichment media mentioned. These organisms grow after subculturing on solid indicator media such as *Salmonella shigella* medium (SS) and a great number of lactose-negative colonies must be examined in order to detect *Salmonella*.

A medium which satisfies both criteria of enrichment has been developed in this laboratory. The use of this medium was responsible for the detection of almost twice as many cases of salmonellosis as when the two other standard enrichment media were used.

Materials

Enrichment Media.—Tetrathionate broth was prepared as follows:

Nutrient broth	...	...	90 ml.
CaCO ₃	...	...	5.0 gr.
Sodium thiosulphate	...	...	5.0 gr.
Ox bile	...	...	5.0 ml.

Each ingredient was sterilized separately by autoclaving and when sterile all ingredients were aseptically added to nutrient broth, followed by 1 ml. of 1:1000 brilliant green and 2 ml. of lugol solution. A modification of this medium with malachite green instead of brilliant green was also used (Wild, 1952).

Sodium selenite broth was prepared according to the method of Hobbs and Allison (1945).

The new enrichment medium magnesium chloride-malachite green required the following:

<i>Solution A.</i> —Bacto tryptone	...	0.5 gr.
NaCl	...	0.8 gr.
KH ₂ PO ₄	...	0.16 gr.
Bi-distilled water	...	100 ml.

Solution B.—For this 40 gr. of MgCl₂ (C.P.) is dissolved in 100 ml. water (it is advisable to dissolve the entire contents of MgCl₂ from a newly opened container according to the formula, as this salt is hygroscopic).

Solution C.—A 0.4% solution of malachite green in distilled water is required.

For use, to each 100 ml. of solution A is added 10 ml. of solution B and 3 ml. of solution C. The final medium is distributed in 5 ml. quantities in test tubes, autoclaved for 20 min., and stored in a refrigerator.

Differential media are SS agar medium (Difco), Kligler iron agar, and urea indol medium (Rappaport and Henig, 1951).

Technique

The stool to be examined was emulsified as a 1:1000 suspension in saline. Three to four drops of this suspension were dropped into 5 ml. of the following enrichment media: tetrathionate, sodium selenite enrichment broth, and the new enrichment medium. After an incubation of 16 to 18 hours a loopful of each of the enrichment media was plated on SS agar. Lactose-negative colonies on SS were inoculated into Kligler's iron agar and urea indol medium (Rappaport and Henig, 1951), and were examined serologically.

Evaluation of the results was based on two criteria: the ratio of positive findings from one enrichment medium to positive findings from another and the presence or absence of contaminants.

During the course of development of the new medium, when various ingredients at various concentrations were tried, essentially the same procedure as described above was followed. We used artificially infected stool suspension by adding to a *Salmonella*-free suspension of faeces a small number (1-100) of *Salmonella paratyphi A*, *Salmonella paratyphi B*, and *S. typhi*, respectively.

Development of the New Medium

Inorganic Salts and Dyes.—The use of hypertonic magnesium chloride in the new medium follows the

on SS agar showed no significant differences. The faecal suspension used for dilutions contained 5×10^7 microorganisms/ml. The results of tests performed as above showed that the degree of multiplication in enrichment medium of *Salmonella* suspended in saline or in faecal suspension was of the same order, and a population of approximately 2×10^7 /ml. was reached from each *Salmonella* inoculated. It was also found that coliforms disappeared and a practically pure culture of *Salmonella* was obtained (Fig. 2b). If 1–5 organisms of *Salmonella* were mixed with 10^6 coliforms in the new enrichment medium then the *Salmonellae* would be isolated.

Quantitative experiments were performed in order to demonstrate the growth of *Salmonella* and inhibition of *E. coli* from a mixture inoculated into the new enrichment medium. Samples of tenfold dilutions of a suspension of *S. paratyphi B* in saline were added to a suspension of *E. coli*, containing 3×10^7 organisms/ml. From these mixtures 0.1 ml. was inoculated into 5 ml. of the new medium. By this means 25, 250, and 2,500 *Salmonellae* were inoculated accompanied by 3×10^6 *E. coli*. From each tube of the new medium

samples were taken after five, 13, and 27 hours' incubation; the samples were diluted in tenfold steps and assayed on SS medium by plating 0.1 ml. of each dilution. As controls two tubes of the new medium were tested, one inoculated with *S. paratyphi B* alone and the other with *E. coli* alone. The number of colonies of *E. coli* growing on SS agar was about half the number which grew on nutrient agar. *S. paratyphi B* gave equal counts on SS and on nutrient agar. The results of one such experiment are graphically represented in Fig. 1. After five hours of incubation there is already an increase in the numbers of *Salmonella*, and colonies could be seen on SS agar in spite of an overwhelming growth of *E. coli*. After 13 hours of incubation in the new enrichment medium, the predominant growth on the plates was *Salmonella*. Even on plates inoculated with a sample of the new medium originally containing one *Salmonella* organism in 0.2 ml., after 13 hours' incubation there were 100 times more *Salmonellae* than *E. coli*. At the end of the logarithmic growth phase, after about 16 hours, *Salmonella* outnumbered *E. coli* by 30,000:1.

The only quantitative comparative results reported on enrichment media were made by Lang (1952, 1954), who mixed various proportions of *Salmonellae* with either *E. coli* or *Proteus* or *A. aerogenes*, inoculated them into several enrichment media and determined the maximum ratio at which *Salmonella* would yet grow. He found that the best enrichment and selective media were those of the Mueller-Kauffman tetrathionate broth. Bierbrauer increased the selectivity of the medium by the addition of 0.01% malachite green and 0.0025% brilliant green. Wild's medium is solid and contains only 0.002% malachite green. In Bierbrauer's medium *Salmonellae* would grow even when inoculated with *E. coli* at a ratio of 1:30,000. This ratio is termed by Lang the "valency coefficient." In the original Mueller-Kauffman medium the valency coefficient is only 3:11. The efficiency of Bierbrauer's medium in relation to *Proteus* and *A. aerogenes* was found by Lang (1952) to be much lower than that in relation to *E. coli*.

FIG. 1.—Growth of *S. paratyphi B* and of *E. coli* from a mixture inoculated into the new enrichment medium (mm.). C1=*E. coli* in presence of *Salmonella*, C2=*E. coli* control (without *Salmonella*). S=*S. paratyphi B* control (without *E. coli*). S1, S2, and S3=*S. paratyphi B* in presence of *E. coli*. Each tube of the new enrichment medium was inoculated with 2.5×10^6 of *E. coli* and 25 (S1), 250 (S2), and 2,500 (S3) organisms of *S. paratyphi B*.

TABLE III
COMPARISON OF ENRICHMENT EFFICIENCY OF THREE MEDIA

Medium	Number of Pathogens Isolated in Group							SI %				
	A	B	C	D	E	G	—	Total	B	C	Others	Total
I	4	16	16	—	1	—	—	37	38.1	27.6	(*)	30.8
II	5	21	31	3	3	—	1	64	50.0	53.4		52.1
III	6	39	53	6	5	1	1	111	92.9	91.0		92.5
I + III	5	24	34	3	3	—	1	70	57.1	58.6		58.3
I + II + III	7	42	58	6	5	1	1	120	100.0	100.0		100.0
I + III	7	41	56	6	5	1	1	117	97.6	96.6		97.5
II + III	7	41	57	6	5	1	1	118	97.6	98.3		98.3

* Not evaluated (too few cases). SI Sensitivity Index.

I = Selenite broth. II = Tetrathionate broth (TTB). III = New medium (MM). A, B, C, D, E, G = *Salmonella* "O" groups (see Table IV).

Using Lang's criteria, the new enrichment medium is superior to Bierbrauer's, and its valency coefficient is of the order of $1:10^5$ – 10^6 , compared with $1:30,000$ in Bierbrauer's medium. This is true both in relation to *E. coli* and to a mixture of all coliforms in a faecal suspension.

Clinical Evaluation.—During the year 1954 stool samples of patients admitted to hospital were routinely tested by the described enrichment procedure in order to detect the presence of *Salmonellae*. All samples were enriched in the three media described. Of 3,391 stools examined, altogether 120 *Salmonellae* were isolated. Their distribution according to frequency of isolation from each enrichment medium is shown in Table III, and the identification of the strains is given in Table IV. According to Gillert (1954) the yield of the isolated pathogens by a given method should be compared to the yield obtained by the largest possible number of bacteriological methods. This was done, and the number of *Salmonella* strains isolated by all three methods employed was taken to be equal to 100%. On this basis all other results were calculated. The ratio of the number of pathogens isolated by a given method to the total number isolated by all three methods was termed the "sensitivity index" (Table III). From the series presented in the table it may be seen that the new enrichment medium permitted the detection of 92% of all cases, while the selenite enrichment broth detected only 31% and the tetrathionate broth 52%. When in addition to the new medium one other enrichment medium, either selenite or tetrathionate, was used, 98% of cases were detected.

The fact that selenite and tetrathionate broths are not suitable for enrichment of certain *Salmonellae* was pointed out by Banwart and Ayres (1953). They studied the growth of six species of *Salmonella* in various enrichment broths and on various selective media. *S. paratyphi* was found to be inhibited by tetrathionate broth and *S. anatum* by selenite. Lang (1954), describing the very promising results obtained on Wild's tetrathionate medium, remarks that this medium is inhibitory to *S. pullorum* and *S. abortus equi*.

Of great importance is the fact that the new enrichment medium effectively prevents the development

of intestinal bacteria accompanying the *Salmonellae*. There were eight times more contaminated cultures on SS agar when plated from selenite broth and four times more when plated from tetrathionate broth, than when the inoculum was taken from the new medium. Its freedom from contaminants is strikingly demonstrated in Figs. 2a and 2b.

Selenite broth was reported by Leifson (1936) to inhibit faecal cocci and enterococci within eight to 12 hours, while *S. typhi* multiplied from the start. Similar results were described by Macierewicz, Strzelecka, and Zaleska (1954). However, *Proteus* and *Ps. pyocyanea* were not inhibited in this medium. The presence of contaminants such as *E. coli*, *Ps. pyocyanea*, *A. aerogenes*, and yeasts in inocula from tetrathionate and selenite broth has been also observed by Biechteler (1952).

Another factor contributing to freedom from contamination, which we find of importance in our en-

TABLE IV
TYPES OF *SALMONELLAE* ISOLATED BY ENRICHMENT PROCEDURE

Pathogens Isolated		Media			
Group	Species	Number	In All Three	In Two	In One*
A	<i>S. paratyphi A</i> ..	7	3	2	2
B	<i>S. paratyphi B</i> .. <i>S. typhi murium</i> ..	42	12	10	20
C	<i>S. paratyphi C</i> .. <i>S. braenderup</i> .. <i>S. newport</i> .. <i>S. montevideo</i> .. <i>S. muenchen</i> .. <i>S. emek</i> .. <i>S. nachsonim</i> ..	58	11	20	27
D	<i>S. enteridis</i> ..	6	—	3	3
E	<i>S. meleagridis</i> .. <i>S. london</i> .. <i>S. taksony</i> ..	5	1	2	2
G	<i>S. poona</i> ..	1	—	—	1
—	<i>S. tel-aviv</i> ..	1	—	1	1
	Total pathogens ..	120	27	38	55

* On new enrichment medium.

FIG. 2a.—Smear from tetrathionate broth on an SS agar plate after 18 hours of incubation. The enrichment broth was inoculated with 3 drops of faecal suspension (1:1,000).

richment procedure, is the use of relatively dilute faecal suspensions. In tetrathionate and selenite broths this dilution allows the growth of coliforms. In spite of this dilution growth of *Salmonellae* in the new medium was practically unrestricted, one organism being sufficient to start growth in presence of 10^6 coliforms. Fig. 3 demonstrates the importance of dilution of faeces before inoculation into enrichment media. Only in inocula containing less than 1:1,000 dilution of faeces, lactose-positive colonies were found. A simple calculation shows that in the case presented in Fig. 3 even 0.1 micrograms of faeces was sufficient to initiate growth of *S. enteritidis* in the new medium.

FIG. 3.—An SS agar plate inoculated from the new enrichment medium. Numbers denote logarithm of dilution of faeces inoculated into the medium. From dilutions 10^{-3} to 10^{-6} (3–6) grew only *S. enteritidis*. Lactose-positive colonies were present in dilutions 1 and 2.

2B

FIG. 2b.—Smear from the new enrichment medium on an SS agar plate. The enrichment medium was inoculated with the same suspensions as in Fig. 2a.

The results presented in Table III demonstrate the superiority of the new enrichment medium over selenite broth and tetrathionate broth. The new medium is, however, not suitable for the isolation of *S. typhi*; this organism is inhibited by malachite green at the concentration employed. When the concentration is lowered, contaminants may develop. We agree with Wundt (1954) that at present selenite broth is the most suitable enrichment medium for *S. typhi*.

Summary

A new enrichment medium for the cultivation and isolation of *Salmonellae* from faeces is described.

The ingredients in this medium, which inhibit the growth of coliform contaminants and permit unrestricted development of *Salmonellae*, are magnesium chloride (4%), and malachite green (0.012%). The medium was found to be superior for enrichment of *Salmonellae*, with the exception of *S. typhi*, to selenite enrichment broth and tetrathionate broth.

An important modification of the inoculation procedure is the use of a 1:1000 suspension of faeces for inoculation of the enrichment medium. This dilution ensures freedom from contamination by coliforms and does not decrease the number of isolations of *Salmonellae*.

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