SHORT ARTICLES

A NEW SELECTIVE MEDIUM FOR THE ISOLATION OF SALMONELLAE OTHER THAN *SALMONELLA TYPHI*

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THE DEVELOPMENT of selective media has greatly improved the isolation of salmonellae from faeces, sewage and food. Several media, such as brilliant-green phenol-red agar (Kristensen, Lester and Jurgens, 1925), the medium of Wilson and Blair (1926, 1927, 1931) and deoxycholate-citrate agar (Leifson, 1935), as modified by Hynes (1942), are still in general use. Nevertheless they are far from ideal (*British Medical Journal*, 1968). Even when carefully prepared and controlled, they often support an abundant growth of *Klebsiella*, *Proteus* or *Pseudomonas* which may obscure the presence of *Salmonella*. Wilson and Blair's (WB) medium, and to a less extent that of Hynes, also suffer the serious disadvantage of varying unpredictably from batch to batch.

The new medium described in this paper largely overcomes these difficulties and appears to be more selective, more reproducible and more reliable for the isolation of salmonellae, other than *Salm. typhi*, than the media in common use for this purpose.

MATERIALS AND METHODS

Bacterial strains. All non-salmonella bacteria and most of the strains of *Salm. typhimurium*, *Salm. enteritidis*, *Salm. paratyphi B* and *Salm. typhi* were from patients in Westminster Hospital. Other salmonella strains were obtained from the National Collection of Type Cultures, from Dr Joan Taylor at the Salmonella Reference Laboratory, and from Dr Joan Davies at the London County Hall Laboratory, both of the Public Health Laboratory Service.

Viable counts. Surface viable counts were performed by the method of Miles, Misra and Irwin (1938).

Media. Selenite F Medium was Oxoid R39 (Oxo Ltd, London). Deoxycholate citrate agar (DCA) was prepared according to Hynes' (1942) modification. Peptone water contained (w/v) peptone (Oxoid L37) 1% and sodium chloride 0.05%, at pH 7.4. Peptone agar was prepared by adding agar 1% (w/v) (New Zealand, Davis Gelatine Ltd, Christchurch, New Zealand).

The new medium is essentially MacConkey's agar, made by the method described, supplemented with 0.17M magnesium chloride, 0.057M calcium chloride, 0.0006M cobalt sulphate, 0.005M sodium thiosulphate and 0.00015M zinc sulphate, with sodium novobiocin 6.1 mg, EDTA 1.9 mg, iron dextran 2.4 mg and Tween 80 200 mg per 100 ml. Throughout, thoroughly clean glassware was used, and solutions were made up in clean sterile glass containers. Water was single-distilled from a "Manesty" still, pH 5 to 6.

Preparation of the new medium

MacConkey's agar base. Bacteriological Peptone (Oxoid L37) 80 g, and Bile Salt (Bacto, Difco Laboratories, Detroit, Cat. No. O278-01, containing approximately 67% sodium taurocholate) 20 g were mixed with 4 litres of water and the mixture allowed to stand for about 20 min. at room temperature. The pH was adjusted to 7.4 with 0.1N NaOH or

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HCl. After the addition of 40 g of agar (New Zealand), the medium was steamed for 40 to 60 min. to dissolve the agar. Lactose {"Analar", British Drug Houses (BDH) Chemicals Ltd, Poole, Dorset BH12 4NN} 40 g, and 30 ml of freshly-prepared 1% aqueous neutral red (George T. Gurr Ltd, 136-144 New King's Road, London SW6) were then added, and mixed well. The completed base medium was distributed in 180-ml amounts, autoclaved at 121°C for 15 min. and stored in a cupboard protected from strong light.

Medium supplements. Supplements A and B are required for addition to the base.

Preparation of stock solutions for supplement A. (a) Magnesium chloride $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Hopkin and Williams Ltd, P.O. Box 1, Romford RM1 1HA, Essex, Analytical Reagent; H & W, AR) 43% (w/v): to 430 g, preferably taken from a new bottle since the crystals are deliquescent, water was added to give a final volume of 1 litre. (b) Calcium chloride $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (BDH or H & W) 31% (w/v): 62 g made up to 200 ml with water. (c) Cobalt sulphate $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (H & W, AR) 2.4% (w/v): 4.8 g with water to 200 ml. (d) Zinc sulphate $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (BDH or AR) 0.5% (w/v): 1.0 g with water to 200 ml. (e) EDTA disodium salt (BDH) 0.4% (w/v): 800 mg with water to 200 ml. (f) Iron dextran, supplied as "Imferon" (sterile) by Fisons Ltd (Bishop Meadow Road, Loughborough, Leicestershire LE11 0RG): each 2-ml ampoule $\equiv$ 100 mg iron. (g) Tween 80 (Koch Light Laboratories, Colnbrook, Slough, Bucks) 10% (v/v): 20 ml Tween 80 were added to 180 ml of hot water (c. 90°C) and mixed while hot.

All the solutions except (f) were pasteurised at 60°C for 30 min. and stored at 4°C. These solutions may be used for up to 3 months. "Imferon" (f) is stored in a box at room temperature up to the expiry date.

Preparation of supplement A from the stock solutions. In the order shown the following were mixed: 100 ml of solution (a); 30 ml of (b); 6 ml of (c); 7.5 ml of (d); 6 ml of (e); and 0.6 ml of (f) by transfer from an opened ampoule with a 1-ml Mantoux syringe. These were mixed well, raised to 56°C for 5 min. and remixed. A final addition of 25 ml of (g) pre-heated at 56°C was made, and mixed well several times by inversion; a final remixing at 56°C for 5 min. was made. Supplement A may be stored for up to 3 months at 4°C. It should be used only after 3 days have elapsed since its preparation.

TABLE I
Growth of intestinal pathogens and commensals on peptone agar; effect of
the addition of magnesium or sodium chloride

Organism	Growth* on		
	blood agar	peptone agar containing magnesium chloride	sodium chloride
<i>Escherichia coli</i> , strain 1	+++	—	+++
<i>E. coli</i> , strain 2	+++	—	+++
<i>Pseudomonas aeruginosa</i> , strain 1	+++	—	+++
<i>Ps. aeruginosa</i> , strain 2	+++	—	+++
<i>Proteus mirabilis</i> , strain 1	+++S	+++NS	+++SI
<i>Pr. mirabilis</i> , strain 2	+++S	+++NS	+++SI
<i>Salmonella enteritidis</i>	+++	+++	+++
<i>Salm. typhimurium</i>	+++	+++	+++
<i>Salm. panama</i>	+++	+++	+++

* After 48 h at 35°C; 1% peptone agar, pH 7.4; 0.26M MgCl_2 ; 0.52M NaCl, a concentration chemically equivalent to, but osmotically stronger than, that of the MgCl_2 . +++ = Heavy growth; — = no growth; S = swarming; SI = swarming partially inhibited; NS = no swarming.

Preparation of supplement B. (1) Sodium novobiocin (Upjohn Ltd, Crawley, Sussex, potency 870 μg per mg); 1.5 g was placed in a sterile glass bottle and 200 ml of sterile distilled water were added with frequent tapping and mixing; the powder may take up to 45 min. to dissolve completely. Suitable modifications are made for batches of different potency. (2) Sodium thiosulphate $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (BDH) 10% (w/v): 20 g were made up with water to 200 ml, pasteurised at 60°C for 30 min., and then allowed to cool to room temperature.

Supplement B was made up in 6-ml amounts by mixing equal volumes of solutions (1) and (2), and stored at once at or below -20°C.

FIG.—Inhibition of growth of bacterial strains by combinations of magnesium chloride and novobiocin in MacConkey's agar; incubation for 21 h at 35°C. The vertical and horizontal dashed lines represent respectively a single concentration of MgCl_2 (0.17M) with varied concentrations of novobiocin, and that of novobiocin (70 μg per ml) with varied concentrations of MgCl_2 . The positions of open symbols indicate the combinations (MgCl_2 constant) needed to reduce the viable counts of the intestinal pathogenic (○) or commensal (□) strains indicated 1000-fold or more in comparison with controls. The closed symbols ●, ■, indicate the combinations (novobiocin constant) giving similarly reduced growth of the same strains. Ec = *Escherichia coli*; Pr = *Proteus mirabilis*; Ps = *Pseudomonas aeruginosa*; Sh = *Shigella sonnei*; Sp = *Salmonella paratyphi* B; St = *Salmonella typhi*; Stm = *Salmonella typhimurium*.

The complete new medium. MacConkey's agar base was selected that had been prepared on a previous day but was not more than 18 days old. The agar was melted by steaming for about 40 min. and transferred to a 56°C-water bath. A 6.0-ml portion of supplement B was thawed rapidly at 56°C and mixed. Supplement A was mixed at 56°C for 5 min. just before use. To the melted agar, 30 ml of supplement A was added, followed by 4 ml of supplement B, mixing well before and after each addition. Immediately, about 10 plates were poured from each bottle. The plates were stored at 4°C for up to 1 week, and dried for about 30 min. on the day of use.

Inoculation of media. Cultures of organisms in the log phase were prepared in peptone water, usually after overnight incubation. When surface viable counts were performed, tenfold dilutions were made in peptone water and plates inoculated with a calibrated 50-dropper pipette. On other occasions, media were inoculated with a standard 5-mm loop that had been immersed in a slightly turbid peptone water dilution of an overnight culture. A routine loop was used for direct inoculation of faeces or for subculture of selenite F cultures.

RESULTS

Development of the selective medium

Effect of magnesium and novobiocin. The effect on bacterial growth of magnesium alone added to peptone agar is shown in table I. High concentrations of magnesium alone in MacConkey's agar also inhibited *Pseudomonas aeruginosa* and *Escherichia coli* much more than *Proteus mirabilis* or salmonellae other than *Salm. typhi*. Novobiocin alone at a concentration of 70 µg per ml in MacConkey's agar inhibited *Pr. mirabilis*. The effects of various concentrations of magnesium chloride in the presence of a single concentration of novobiocin and of various concentrations of novobiocin in the presence of a single concentration of magnesium chloride are shown in the figure.

A combination of 0.21M magnesium chloride and novobiocin 70 µg per ml suppressed the growth of *Ps. aeruginosa*, *E. coli* and *Pr. mirabilis* while allowing full growth of *Salm. typhimurium* and *Salm. paratyphi B*. It was not possible to select a combination of these substances that would permit the growth of *Sh. sonnei* and *Salm. typhi* while inhibiting the non-pathogenic organisms. In this paper the term *Salmonella* is taken to exclude *Salm. typhi*.

On the complete new medium lacking only magnesium chloride, the viable counts of the strains of *Salm. typhi* and *Sh. sonnei* were both reduced 10⁶-fold in comparison with those of the controls. When 0.17M magnesium chloride was added, the medium became complete and on this the viable count of the *E. coli* strain was reduced by a factor of 10⁵, and the counts of the strains of *Ps. aeruginosa*, *Pr. mirabilis*, and *Klebsiella aerogenes* by a factor of 10⁶, while the counts of the *Salm. paratyphi B* and *Salm. typhimurium* strains were not reduced at all. The counts of these latter salmonellae were not reduced until the magnesium-chloride concentration was increased to above 0.25M; at 0.28M the counts were reduced by a factor of 1000 in comparison with those of the controls.

Cobalt. The addition of cobalt sulphate 0.21M to MacConkey's medium reduced the viable count of *E. coli*, *Ps. aeruginosa*, *Salm. enteritidis*, and *Pr. mirabilis* more than 10⁹-fold. Cobalt sulphate (0.0006M) in MacConkey's agar containing 0.21M magnesium chloride was selectively toxic to *E. coli* and *Pr. mirabilis*. Growth of *Ps. aeruginosa* and of *Salm. enteritidis* strains was improved by cobalt, and generally the diameter of salmonella colonies was about twice that of the few colonies of *E. coli* and *Pr. mirabilis* that appeared. When sodium novobiocin 70 µg per ml was added to this medium, *Proteus*, *E. coli* and *Pseudomonas* were all totally suppressed, while salmonellae grew with unreduced viable counts and with larger colonies than on a parallel medium without cobalt.

The medium with a MacConkey's base containing 0.21M magnesium chloride, 0.0006M cobalt sulphate and sodium novobiocin 70 µg per ml gave excellent results for some time experimentally and with clinical specimens (Shanson, 1968). Further experience, however, revealed that some batches failed to support the growth of certain salmonellae, particularly *Salm. panama*. It was thought that this was attributable to batch variation of the bile salts. The medium was further modified in an attempt to reduce this variation and if possible to improve the selectivity.

Calcium. Substituting calcium for magnesium at 0.21M did not significantly affect the viable counts of *Salmonella*, *Proteus*, *Pseudomonas* or *Escherichia* on the MacConkey's base with 0.5% taurocholate, novobiocin 70 µg per ml and 0.0006M cobalt sulphate. But selective growth of salmonellae appeared best with 0.07M calcium chloride and 0.14M magnesium chloride and this mixture was used in the subsequent work on development of the new medium.

Bile salts and other surface-active agents. Omission of bile salt allowed growth of *E. coli*. Substitution of commercial bile salt by the purified sodium salt of glycocholic, glycodeoxycholic, glycochenodeoxycholic, chenodeoxycholic, taurodeoxycholic, taurochenodeoxycholic, cholic, glycohyodeoxycholic or dihydrocholic acid was less satisfactory for salmonella selection than pure sodium taurocholate or the commercial bile salts. The latter were preparation no. L55 of Oxoid or no. 0278-01 of Difco, both of which are mainly sodium taurocholate.

A surface-active agent was sought that could either replace the bile salt or be used in conjunction with it to minimise the effect of batch variation obtained with commercial bile salts. None of the following agents was sufficiently selective when used at a 1 % concentration in the experimental media in place of bile salt: propionate, valerate, isovalerate, caproate, palmitate, oleate, stearate, lauryl sulphate, cetyl sulphate, "Teepol", Tween 40, and Tween 80. When tested in combination with 1 % commercial sodium taurocholate, one of these—Tween 80—increased the colony diameters of salmonellae by 0.1–0.3 mm. At the same time, however, depending on the ratio of Tween 80 to bile salt, there was an increase in

TABLE II

Selectivity of new medium and of deoxycholate citrate agar (DCA) for intestinal bacteria

Group*	Species	Number of strains		
		tested on both media	growing† on	
			new medium	DCA
1	<i>Salmonella paratyphi B</i>	7	7	5
	<i>Salm. typhimurium</i>	8	8	7
	<i>Salm. panama</i>	8	8	8
	<i>Salm. enteritidis</i>	4	4	4
	<i>Salm. agona</i>	2	2	2
	<i>Salm. virchow</i>	4	4	4
	<i>Salm. stanley</i>	4	4	4
	<i>Salm. dublin</i>	4	4	4
	<i>Salm. montevideo</i>	4	4	4
	<i>Salm. indiana</i>	2	2	2
	<i>Salm. oranienburg</i>	2	2	2
	<i>Salm. bredeney</i>	1	1	1
	<i>Salm. brandenburg</i>	1	1	1
	<i>Salm. newport</i>	1	1	1
	<i>Salm. infantis</i>	1	1	1
	<i>Salm. heidelberg</i>	1	1	1
	<i>Salm. minnesota</i>	1	1	1
2	<i>Escherichia coli</i>	20	4	7
3	<i>Klebsiella aerogenes</i>	20	2	17
	<i>Proteus mirabilis</i>	20	0	15
	<i>Pseudomonas aeruginosa</i>	20	0	20
4	<i>Shigella sonnei</i>	3	0	3
	<i>Alkaligenes</i> sp.	2	0	2
	<i>Alkalescens</i> sp.	2	0	2
	<i>Hafnia</i> sp.	2	0	2
	Other non-lactose fermenters‡	23	6	23
5	<i>Salm. typhi</i>	3	0	3
	<i>Salm. paratyphi A</i>	2	0	2
	<i>Salm. paratyphi C</i>	2	0	2
	<i>Salm. choleraesuis</i>	1	0	1

* Species grouped according to the results obtained with the two media. *Group 1*: all strains growing with unreduced viable count on the new medium, with colonies of about 0.5-mm diameter and most of them growing on DCA; *group 2*: 20% of strains growing on the new medium and rather more on DCA; *group 3*: few strains growing on the new medium, but most growing on DCA; *group 4*: all strains growing on DCA, but few on the new medium; *group 5*: salmonellae growing on DCA, but suppressed on the new medium.

† Growth after 21 h at 35°C.

‡ Strains growing on DCA: not *Salmonella*, *Shigella*, *Proteus* or *Pseudomonas*; not further identified.

the viable count and colony size of some lactose-fermenting colonies, including *E. coli*. Selection for *Salmonella* appeared best at a ratio of Tween 80 to commercial sodium-taurocholate bile-salt of 1:2.5.

Autoclaving bile salts affected their selective properties in the experimental medium. When bile salts were added as pasteurised solutions, Oxoid no. L55 gave a better result than did Difco no. 0278-01. However, the Difco bile salt appeared preferable if the medium were to be autoclaved as part of the usual method for preparing MacConkey's agar.

Iron, zinc and thiosulphate. On the modified medium containing Tween 80 0.2%, Difco bile salt 0.5%, 0.14M magnesium chloride, 0.07M calcium chloride, novobiocin 70 µg per ml and 0.0006M cobalt sulphate, some *E. coli* strains grew moderately well and further selective agents were sought. Tests with various combinations of cations and anions including lithium, sodium, potassium, zinc, manganese, iron, copper, chloride, acetate, sulphate, nitrate, and thiosulphate showed that three appeared useful: iron, zinc and thiosulphate. The small but definite beneficial effect of iron could not be reproduced with ferrous sulphate or ferrous citrate, but iron dextran at a final concentration of 2.4 mg per 100 ml gave good results. The addition of zinc sulphate at 0.00015M and thiosulphate at 0.005M inhibited some of the *E. coli* strains without reducing the viable counts of salmonellae.

EDTA and pH. EDTA was added on theoretical grounds to stabilise cations, although experimentally it appeared neutral. The optimum pH for selectivity was about 6.9.

Magnesium and calcium in the whole medium. When all reagents were finally included in the new medium, a small adjustment in the calcium:magnesium ratio was found necessary for best selectivity. The molarities finally adopted were: magnesium chloride 0.17 and calcium chloride 0.057.

Experimental behaviour of the new medium

The behaviour of the medium with an inoculum of about 10^6 bacteria in peptone water is summarised in table II. From this it can be seen that *Salm. paratyphi B* and many of the serotypes of *Salmonella* associated with food poisoning (Vernon, 1970) grew well. Compared with DCA, colony sizes were somewhat smaller. Four species, none of which commonly causes food poisoning, were inhibited. The selectivity was clearly better than that of DCA for all salmonellae tested other than these four. The new medium appeared to inhibit at least as many *E. coli* strains as did DCA, and was much more inhibitory than DCA to *Proteus*, *Klebsiella*, *Pseudomonas*, *Shigella* and numerous miscellaneous non-lactose fermenters.

In the course of this work a set of organisms was selected that proved to be of use in predicting the performance of different batches of medium. This set included strains known to grow: *Salm. paratyphi B*, *Salm. typhimurium*, *Salm. panama* and *Salm. dublin*; and others known to be inhibited: *Pr. mirabilis*, *Klebsiella*, *E. coli* and *Ps. aeruginosa*. The growth characteristics of these strains varied very little with different batches of the new medium.

Direct inoculation of faeces containing *Salm. typhimurium* or *Salm. paratyphi B* normally yielded a pure or almost pure growth of *Salmonella*, whereas parallel cultures on DCA often showed a profuse growth of *Klebsiella*, *Proteus* or *Pseudomonas*. Subcultures from selenite F behaved essentially similarly except that faeces from control individuals gave growth even less often than after direct inoculation.

Saline suspensions of *Salmonella* are somewhat less easily made from the new medium than from less selective media, but are almost always smooth and specifically agglutinable by sera on the slide. Subculture of doubtful colonies on to MacConkey's agar readily allows salmonellae to be distinguished by agglutination from colonies of *Escherichia* or other genera that occasionally grow on the new medium and may resemble salmonellae.

DISCUSSION

Magnesium chloride was found to be the most selective of a number of salts tested by Rappaport (Rappaport, Konforti and Navon, 1956) for salmonellae in liquid media, and was one of the main agents used in Rappaport's liquid enrichment medium. A selective activity of the magnesium ion is confirmed by the results shown in table I. Magnesium and sodium

salts in chemically equivalent amounts have markedly different inhibitory effects. No record has been found of its previous use in high concentration as a selective agent in a solid medium. These authors did not mention any inhibitory effect of magnesium chloride against *Ps. aeruginosa*.

Novobiocin has previously been used to inhibit growth of *Proteus* in tetrathionate broth (Jeffries, 1959) and in a solid medium to inhibit spreading of *Proteus* (Pohl and Thomas, 1966).

Cobalt and zinc have not previously been employed in selective media for salmonellae. Iron-dextran solution, marketed for the treatment of iron-deficiency anaemia, does not appear to have been used previously in the preparation of media. As a convenient and reproducible source of sterile iron, it might have a use in preparing other media where the inclusion of iron is thought to be desirable.

Bile salt employed in the new medium introduces the possibility of batch variation, but with the presence of Tween 80 in the final formula this has not so far proved to be a problem. Different batches of DCA may vary greatly, and at times even very carefully prepared batches may be greatly inhibitory to salmonellae. This contrasts with the new medium which has appeared substantially more reproducible. Nevertheless the behaviour of medium containing a new batch of bile salt must be tested experimentally and any necessary adjustment made before it is used routinely.

WB medium was not compared with the new medium. Such a comparison is desirable, since excellent results have been claimed for WB medium (McCoy, 1962; McCoy and Spain, 1969) in the isolation of salmonellae in general, in addition to the typhoid bacilli for which it is said to be "pre-eminently" suitable (Harvey, 1956). However, the difficulty of preparing satisfactory batches of WB medium is considerable in a laboratory that is not using it continuously. Theoretically WB medium would be expected to be less selective than the new medium for salmonellae other than *Salm. typhi* in specimens containing *Proteus* or *Pseudomonas*. The fact that WB medium is liable to vary from batch to batch, and with the age of the plates, puts it at some disadvantage in comparison with the new medium.

Though good results have been obtained with selenite F medium for enrichment before subculture of the new medium, other enrichment media, such as tetrathionate broth or Rappaport's medium, might give better results and a trial to determine this might prove worth while. An enrichment medium composed of the agents used in the new medium in liquid form might well prove best of all.

The new medium is likely to be of most value in the examination of large numbers of faeces for salmonellae other than *Salm. typhi* during outbreaks of salmonella food poisoning or paratyphoid B infection. After a preliminary test had shown that the salmonella strain implicated in a particular outbreak would grow satisfactorily, a complete change to the new medium might be contemplated. Used in conjunction with selenite F it should save considerable time and materials by avoiding the need to examine the large number of non-pathogenic non-lactose fermenters that grow on DCA. At the same time one would expect the greater selectivity to lead to more salmonella isolations.

Only in laboratories that normally isolate salmonellae from many sporadic cases might it be worth while to use the new medium as a routine in parallel with DCA or another selective medium.

It could be reasonably argued that any additional medium increases the number of salmonella isolations, but the greater its selectivity the more likely is it to be effectively complementary. Perhaps the most practicable measure for some laboratories would be to use the new medium in parallel with DCA routinely as an extra plating medium after selenite F enrichment. But the isolation of salmonellae from foods, water and sewage should also be facilitated by the new medium, especially from specimens in which Gram-positive organisms, *Proteus*, or pseudomonads are abundant.

SUMMARY

A medium consisting of a MacConkey's base with added magnesium, calcium, cobalt, zinc, thiosulphate, novobiocin, iron dextran, Tween 80 and EDTA has been found highly

selective for *Salmonella paratyphi B* and most of the food poisoning salmonellae that commonly occur in Britain. Nearly all the normal faecal flora is inhibited.

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