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Comparison of MacConkey broth, Teepol broth and glutamic acid media for the enumeration of coliform organisms in water

By the Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies*

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INTRODUCTION

A chemically defined medium based on glutamic acid was first advocated by Folpmers (1948) in Holland for the enumeration of the coliform group of bacteria in water. Burman & Oliver (1952) at the Metropolitan Water Board carried out trials using some modifications of media and techniques. A trial of these techniques was carried out by the Public Health Laboratory Service (1958), which reached similar conclusions. These were that glutamic acid medium containing glucose gave good agreement with MacConkey broth in 24 hr. but too many false positive results in 48 hr. With a lactose modification more *Escherichia coli* were isolated in 48 hr. but it was too inhibitory for other coliform organisms. MacConkey broth itself was shown to have various defects in performance.

The lactose medium was then further improved by Gray (1959), who increased the pH and added sodium formate to increase gas production. Further modifications were made at the Metropolitan Water Board (MWB) (Windle Taylor, 1959–60; 1961–62). The concentration of lactose was increased and the phosphate decreased, and the modified medium was adopted in place of MacConkey broth for all routine samples examined by the multiple tube method, from January 1962.

Simultaneously and independently Gray also made further modifications resulting in the publication of an improved formate lactose glutamate medium (Gray, 1964). This was a more nutrient medium containing additional amino acids, growth factors, mineral salts and ammonium chloride instead of the unstable ammonium lactate. Ammonium chloride had already been incorporated in the MWB medium on Gray's recommendation.

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The Water Research Association (WRA) also carried out a simultaneous and independent investigation of glutamate media resulting in the publication of yet another recommended modification (Collingwood, 1964). This had been compared only with the MWB medium because Gray's improved medium was not then available. The WRA medium included one additional amino acid, two growth factors but not those used by Gray, changes in the mineral composition, a reduction in lactose and an increase in glutamic acid. The composition of the three media, MWB, WRA and Gray's improved version are given in Table 1.

Table 1. *Modifications of glutamic acid media*

	Burman MWB	Gray PHLS	Collingwood WRA
Lactose	20 g.	10 g.	5 g.
L(+)glutamic acid	5 g.	5 g.	10 g.
L-cystine	—	0·02 g.	0·05 g.
L(−)aspartic acid	—	0·024 g.	—
L(+)arginine	—	0·02 g.	—
Nicotinamide	—	—	1 ml. of 0·1 % aq. soln.
Riboflavine	—	—	1 ml. of 0·002 % aq. soln.
Thiamin	—	1·0 mg.	—
Nicotinic acid	—	1·0 mg.	—
Pantothenic acid	—	1·0 mg.	—
$MgSO_4.7H_2O$	—	0·2 g.	1 ml. of 1 % aq. soln.
KCl	—	—	9 g.
K_2HPO_4	1 g.	1 g.	1 g.
HCOONa	0·25 g.	0·25 g.	—
$HCOONH_4$	—	—	2 ml. of 25 % aq. soln.
NH_4Cl	2·5 g.	2·0 g.	—
$CaCl_2$	—	0·2 g.	—
Ferric citrate scales	—	0·1 g.	—
pH	6·7	6·7	6·7
Brom-cresol purple 1 % ethanolic soln.	1 ml.	1 ml.	1 ml.
Distilled water	1000 ml.	1000 ml.	1000 ml.

The above formulae are for single strength media. Double strength media are normally used with 50 ml. and 10 ml. water samples.

While all these glutamate media were under investigation, there was a parallel development designed to eliminate some of the variability of MacConkey broth. Jameson & Emberley (1956) recommended the substitution of Teepol for the very variable bile salts in MacConkey broth. In order to obtain a standard product it is now necessary to specify Teepol 610 (BDH Ltd). This is a 34 % aqueous solution of the sodium salts of straight chain secondary alkyl sulphates containing 8 to 10 carbon atoms in the side chain. Other workers who have compared this medium with MacConkey broth have all reported favourably (Jebb, 1959; Windle Taylor, 1959–60).

The Public Health Laboratory Service Standing Committee on the Bacteriological Examination of Water Supplies was formed in 1964. Among its terms of reference was 'to examine the application of new techniques to routine purposes'.

One of their most urgent tasks was therefore to examine the new techniques outlined above.

With this in view, a trial in eleven laboratories was carried out comparing MacConkey broth with Teepol broth and three modifications of glutamic acid medium.

MATERIALS AND METHODS

Coliform organisms

Throughout this paper the term coliform organisms refers to all Gram negative rod-shaped bacteria, capable of production of acid and gas from 1 % lactose peptone water in 48 hr. at 37° C. The term therefore includes *Esch. coli*.

Media

It was acknowledged from the start that MacConkey broth, the existing standard medium recommended in Report No. 71 (Report 1956) for enumerating coliform organisms and *Esch. coli* in water, was itself variable in behaviour because of variability in properties of both bile salts and peptone. To avoid complications due to this factor, a single batch of Oxoid dehydrated MacConkey broth was distributed to all participating laboratories for use as a standard. In addition each laboratory made MacConkey broth as required from the normal material available to them. Teepol broth was made in each laboratory by the method recommended by Jameson.

The single strength Teepol medium was prepared from peptone, 20 g.; NaCl, 5 g.; Teepol 610 (BDH Ltd), 1 ml.; lactose, 10 g.; phenol red (0·4 % solution), 2·5 ml.; distilled water to 1000 ml. The pH was adjusted to give a final pH of 7·5 after autoclaving at 115° C. for 15 min. The peptone was the same as that used for the laboratory-prepared MacConkey broth and was not the same in all laboratories. This medium differs from Jameson & Emberley's (1956) original formula by the specification of Teepol 610 as already explained and by the use of phenol red instead of bromocresol purple. Teepol modifies the pH range over which most indicators change colour, but phenol red is an exception. Furthermore, phenol red has not been shown to have any toxic effects on any organisms at normal indicator concentrations.

The three glutamic acid media outlined in Table 1 were prepared in dehydrated form especially for the Committee by Oxoid Ltd, partly to ensure standardization, partly because some laboratories had not the facilities to prepare a variety of complex media and partly because it was necessary to determine whether dehydrated media could be prepared which would give satisfactory results.

In the preparation of Gray's modified medium (Gray, 1964) a precipitate occurs on adding $CaCl_2$ solution to the double strength medium. Analysis has shown that this precipitate is composed of phosphates of calcium, iron and magnesium. It would be preferable to avoid a precipitate by adjusting the proportions of the mineral salts so that they remained in solution. This has been done in the Oxoid dehydrated medium. However, on analysis of Gray's medium prepared in the laboratory, the actual concentrations of mineral salts remaining in solution were

found to be different from those in the Oxoid medium. Furthermore, the proportions of the mineral salts remaining in solution varied widely on different occasions even though the same general preparation procedure was used. The results of some analyses are compared in Table 2.

In the method of preparation described by Gray, most of the iron and calcium are precipitated as phosphate, whereas in the Oxoid medium the original quantity of iron added by Gray is retained in solution mainly by reducing the concentration of phosphate. It is doubtful, however, whether this is the best way of avoiding a precipitate. Such a marked reduction in phosphate, in addition to reducing the buffering capacity of the medium, would probably reduce growth more than a reduction in iron and almost certainly more than a reduction in calcium. Furthermore, with all but very soft waters an appreciable amount of calcium would be added with the water sample.

Table 2. *Mineral composition of Gray's glutamate medium (double strength) in g./l.*

	Minerals originally added (Gray, 1964)	Minerals remaining in solution after precipitation by Gray's method (range)	Minerals contained in Oxoid medium
K_2HPO_4	2	1·4–1·8	0·6
$MgSO_4.7H_2O$	0·4	0·2–0·4	0·2
Ferric citrate	0·2	0·001–0·05	0·2
$CaCl_2$	0·4	0·02–0·15	0·2

In Gray's original work with a single strength medium all the minerals were retained in solution. Precipitation occurred only on preparation of a double strength medium. Furthermore, the advantage that he found from adding iron and calcium was obtained originally, using suspensions of pure cultures stored in deionized water. Calcium was, therefore, likely to have been a more significant factor than in a water sample naturally containing calcium.

It is suggested, therefore, that, in order to avoid a precipitate with the double strength medium, a mineral composition should be chosen within the analyses obtained with Gray's method of preparation, but with the highest possible phosphate concentration, followed by the highest possible iron concentration, the calcium being reduced to very low concentration if necessary to avoid a precipitate. These variations in composition were not appreciated until after completion of the trials and are still under investigation.

Water samples

Samples of water from a wide range of sources were required including samples likely to contain attenuated, resistant or damaged organisms. Earlier work had shown that marginally chlorinated water containing some surviving organisms would probably accentuate differences between the methods under review. As

such samples are not normally available, some samples were specially treated by some of the laboratories.

For this purpose, samples of polluted water likely to contain between 100 and 100,000 coliform organisms per 100 ml. were collected in Winchester quart bottles. These were kept at 4° C. overnight in the laboratory. The following morning the samples of water were filtered to remove particulate matter which might cause irregular results. Four ml. of 0·38 % solution of NH_4Cl was added to 2 l. to give an ammonia nitrogen concentration of at least 2 mg./l. Sufficient ice cubes prepared from water free from coliform organisms were added to keep the temperature as near 0° C. as possible during chlorination. Twenty ml. of a hypochlorite solution containing approximately 100 mg./l. of available chlorine was then added to give a chlorine concentration of approximately 1 mg./l. and the water was left to stand for 15–60 min. Any residual chlorine was then neutralized by the addition of 1 ml. of a 3 % solution of sodium thiosulphate sterilized in the autoclave. The chlorination time was chosen by trial and error so that the final sample of water would give some positive and some negative tubes in a 48 hr. test.

Unchlorinated water samples from various sources were examined by all participating laboratories. These included samples stored in bottles by some laboratories.

Methods of recording results

Either one 50 ml., five 10 ml. and five 1·0 ml. volumes or three five-tube ten-fold dilutions were set up for each sample with each of the six media and incubated directly at 37° C. without prior warming. They were examined after approximately 18, 24 and 48 hr. The amount of acid and gas produced was recorded as follows:

A 1 Minimum detectable acidity.
A 2 Acidity between A 1 and A 3.
A 3 Complete acidity.
G 1 Gas on tapping or shaking only.
G 2 Visible gas bubble, insufficient to fill concavity of inner tube.
G 3 Sufficient to fill concavity or more.

All tubes showing less than A 3 and G 3 in 18 and 24 hr. were subcultured and then returned to the incubator for reading at 24 and 48 hr.; they were not subcultured again. All tubes showing A 3 and G 3 in 18 and 24 hr. were assumed to remain so in 24 and 48 hr.

All presumptive positive tubes were subcultured into brilliant green bile broth (BGB) of a single batch and into peptone water, usually 2 % Oxoid tryptone, for indole production. These were incubated in a water bath at $44 \pm 0{\cdot}25$° C. for 24 hr. *Esch. coli* was recorded as present in all tubes giving positive results in both tests. All tubes negative after culture in BGB at 44° C. were examined for coliform organisms of any kind. This was done by plating from the original positive presumptive tube to MacConkey agar and incubating at 37° C. To save time all 24 and 48 hr. presumptive positive tubes were usually subcultured in this way. A growth of typical coliform colonies in 24 hr. was accepted as confirmation; doubtful colonies were subcultured to lactose peptone water. Acid and gas production in

48 hr. at 37° C. was taken as confirmation of coliform organisms. The final results for each primary tube were recorded individually, for each acid and gas category for each time interval, so that results could be compared on the basis of numbers of tubes or most probable numbers of organisms.

RESULTS

Methods of comparison

All comparisons between media have been made on the basis of numbers of positive tubes obtained. This comparison involves a bias in favour of laboratories which examined the greatest number of samples. Results from all the individual laboratories, which examined sufficient samples to be individually compared, have therefore been discussed separately but detailed results have not been presented. As all positive reactions have been counted there is also a bias in favour of samples giving large numbers of positive tubes. This tends to mask the differences between the methods. Selection of the higher dilutions only for comparison, that is those giving some negative tubes, would have accentuated the differences.

Glutamate media

All participating laboratories soon found that there were marked differences in performance between the three modifications of glutamic acid media. The results for all laboratories are summarized in Table 3. This shows the outstanding superiority of Gray's modification over the other two glutamic acid media. This superiority was significant for all types of samples, for both coliform organisms and *Esch. coli*, for all degrees of acid and gas production and for all time intervals. In other words Gray's modification not only gave higher results than the other glutamate media, both for coliform organisms and *Esch. coli*, but it gave them in a shorter time with greater production of acid and gas, thus making positive reactions more easily recognizable.

The early recognition of the superiority of Gray's medium led to the decision to omit the MWB and WRA modifications from subsequent trials. Not only were the MWB and WRA media less productive of positive results than Gray's medium, but they were also slower and significantly less productive of coliform organisms than MacConkey broth. The yield of *Esch. coli* in 48 hr. was not significantly different. In this respect the results differed from previous trials with these media (Collingwood, 1964; Windle Taylor, 1959–60). This may be related to the use of dehydrated media.

Although Gray's medium gave more false positive results than the other glutamate media, this was not serious, since it gave fewer than the MacConkey broths. The superiority of Gray's medium was found by all laboratories. For unchlorinated waters the MWB medium gave the fewest positive results at 24 hr. but showed improvement at 48 hr. This again applied to coliform organisms and *Esch. coli*, for all degrees of acid and gas production and for all time intervals, although there was some variation between individual laboratories.

Table 3. *Comparison of laboratory-prepared and Oxoid MacConkey broths with MWB, WRA, and Gray's glutamate media*

		% of all +ve reactions. False positive results			Number of tubes yielding								
					False positive reactions			Coliform organisms			*Esch. coli*		
		18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
	Unchlorinated samples (no. of tubes examined 1036)												
A 1 G 1 or more	Oxoid MacConkey	0·7	2·5	10·7	4	15	64	363	485	600	277	252	266
	Lab. MacConkey	1·0	3·1	9·0	6	18	52	375	493	580	220	248	253
	MW glutamate	0·2	0·2	3·8	1	1	18	107	306	468	97	206	263
	WRA glutamate	0·0	0·6	5·7	0	3	30	147	315	525	109	212	279
	Gray glutamate	0·3	0·9	9·4	2	6	60	270	446	635	209	303	368
A 2 G 2 or more	Oxoid MacConkey	0·3	1·2	8·1	2	7	48	336	446	593	224	246	266
	Lab. MacConkey	0·9	1·6	7·1	5	9	40	327	432	560	213	247	251
	MWB glutamate	0·0	0·0	2·9	0	0	13	67	259	444	66	179	256
	WRA glutamate	0·0	0·4	3·1	0	2	15	90	261	485	73	180	274
	Gray glutamate	0·0	0·2	7·6	0	1	48	216	402	630	158	283	368
	Chlorinated samples (no. of tubes examined 1375)												
A 1 G 1 or more	Oxoid MacConkey	0·6	3·2	10·8	5	26	87	348	610	805	284	419	447
	Lab. MacConkey	2·9	6·0	15·4	24	49	126	344	616	818	279	388	428
	MWB glutamate	0·0	0·5	7·9	0	3	52	39	310	662	34	248	445
	WRA glutamate	0·0	0·1	6·1	0	1	45	35	269	678	34	232	449
	Gray glutamate	0·0	0·3	11·4	0	3	102	157	561	895	141	456	595
A 2 G 2 or more	Oxoid MacConkey	0·5	1·4	7·2	4	11	56	266	553	780	230	404	445
	Lab. MacConkey	2·4	4·4	11·4	19	35	91	287	564	801	257	381	428
	MWB glutamate	0·0	0·0	1·7	0	0	11	24	215	632	23	181	442
	WRA glutamate	0·0	0·0	2·6	0	0	16	18	183	623	17	167	446
	Gray glutamate	0·0	0·1	7·4	0	1	64	101	479	870	96	393	592

MacConkey broth

Similarly, the Oxoid MacConkey broth was compared simultaneously with laboratory-prepared MacConkey broths. Results for all laboratories are also summarized in Table 3. There is fairly close agreement between the two media. Comparison of each pair of figures shows that the Oxoid medium gave more positive results than the laboratory MacConkey broth under most circumstances, though most of these differences are not significant. The Oxoid medium also gave significantly fewer false positive results with chlorinated samples. It was found that two laboratories obtained consistently lower results with laboratory-prepared medium. One of these laboratories had deliberately used bile salts from a nationally available source, which conformed to the normal requirements for use in MacConkey broth but had given unsatisfactory results with membrane filters. Consistently better results with laboratory-prepared media were given by two other laboratories.

It can be concluded, therefore, that the Oxoid MacConkey broth was a satisfactory standard for comparison with other media, as it gave results within the range of variability encountered in laboratory-prepared media, and with numbers of positive results more often above than below those with laboratory-prepared media.

Gray's glutamate medium compared with MacConkey broth

For the remainder of the trials laboratory-prepared MacConkey broth was therefore omitted and only three media—Oxoid MacConkey broth, Teepol broth and Gray's modified glutamic acid medium—were retained for comparison. The results for all laboratories are summarized in Table 4. This shows clearly that at 48 hr. Gray's medium gave significantly higher numbers of positive results ($P \leqslant 0{\cdot}05$) for coliform organisms and *Esch. coli* than the other two methods, with both chlorinated and unchlorinated waters for all degrees of acid and gas production. This was consistent for all laboratories for *Esch. coli*. At two laboratories Gray's medium gave lower coliform counts than MacConkey broth with unchlorinated samples and at two other laboratories Gray's medium gave lower coliform counts than MacConkey broth with chlorinated samples. These results suggest that the increased numbers of coliform organisms obtained with Gray's medium are probably due mainly to increases in the counts of *Esch. coli*. In fact the increase in numbers of isolations of *Esch. coli* is greater than that of coliform organisms although the former are included in the latter. It does not follow, however, that coliform organisms other than *Esch. coli* would not be so readily isolated with Gray's medium. It merely signifies that where *Esch. coli* and other coliform organisms occur together, growth of the *Esch. coli* has not been suppressed by the other coliform organisms. This has been verified at one laboratory which examined a special series of samples containing moderate numbers of coliform organisms but very few *Esch. coli*. These results will be described later.

In 24 hr. Gray's medium gave significantly more positive results for coliform organisms and *Esch. coli* in unchlorinated waters. The differences are not significant for chlorinated samples. Similar results for *Esch. coli* were obtained by all laboratories. Four laboratories obtained lower coliform counts with Gray's medium than

Table 4. *Comparison of MacConkey Broth, Teepol Broth and Gray's glutamate medium*

		% of all +ve reactions. False positive results			Number of tubes yielding								
					False positive results			Coliform organisms			*Esch. coli*		
		18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
	Unchlorinated samples (no. of tubes examined 3409)												
A 1 G 1 or more	Oxoid MacConkey	0·7	2·1	10·3	11	35	172	1119	1354	1676	804	837	854
	Teepol	0·9	4·4	10·1	15	77	176	1121	1475	1736	788	869	894
	Gray glutamate	0·1	0·5	8·2	2	10	164	951	1403	1869	790	1047	1145
A 2 G 2 or more	Oxoid MacConkey	0·4	1·3	8·6	6	22	141	982	1248	1637	771	829	853
	Teepol	0·1	2·5	9·0	1	42	153	957	1360	1700	729	866	893
	Gray glutamate	0·0	0·1	6·9	0	2	127	707	1262	1830	618	1000	1140
	Chlorinated samples (no. of tubes examined 1441)												
A 1 G 1 or more	Oxoid MacConkey	0·4	2·8	10·3	3	23	85	360	632	827	290	427	457
	Teepol	0·2	1·6	6·0	2	14	51	330	660	852	268	428	456
	Gray glutamate	0·0	0·4	11·5	0	4	106	173	581	924	152	458	605
A 2 G 2 or more	Oxoid MacConkey	0·2	1·1	6·0	2	9	48	275	573	804	236	413	454
	Teepol	0·1	0·8	4·5	1	7	40	224	593	828	202	410	451
	Gray glutamate	0·0	0·1	7·9	0	1	71	116	475	899	104	413	602

with MacConkey broth with unchlorinated samples and five laboratories did so with chlorinated samples.

In 18 hr. Gray's medium gave significantly fewer positive results than the other media under nearly all circumstances. This was found consistently by all laboratories except two where Gray's medium gave more positive results than MacConkey broth for both *Esch. coli* and coliform organisms, especially where low degrees of acidity and gas production were included, with both chlorinated and unchlorinated samples.

Teepol broth compared with MacConkey broth

The Teepol medium gave significantly better results for coliform organisms in 24 and 48 hr. than MacConkey broth with chlorinated and unchlorinated samples and for *Esch. coli* in 24 and 48 hr. with unchlorinated samples. For *Esch. coli* in chlorinated samples the positive results were slightly lower but the difference is not significant. In 18 hr. the only significant differences between Teepol and MacConkey broths were with chlorinated samples at A 2 G 2, Teepol giving fewer positive results. In 18 hr. Teepol gave consistently more positive results than Gray's medium. Again some variation in performance with Teepol broth occurred in individual laboratories, particularly at 18 hr.

False positive reactions

The number of false positive reactions was negligible in Gray's medium in 18 and 24 hr. They were of greater but still low frequency in Teepol and MacConkey broth. At 48 hr. there was little difference between the three media with unchlorinated samples but with chlorinated samples Teepol gave significantly fewer false positive results than the other media.

The proportions of false positive reactions, however, varied widely in different laboratories and different media. In 48 hr. the false positive results for different media in different laboratories ranged from 0 to 16%. This, of course, reflects variation in the bacterial flora other than the coliform group, in water samples from different parts of the country. In MacConkey broth *Clostridium perfringens* (*welchii*) or mixtures of organisms have usually been reported as the most frequent causes of false positive results. In glutamate media, *Bacillus polymyxa* and *B. macerans* are the commonest causes. *Cl. perfringens* does not usually grow in glutamate media and *B. polymyxa* and *B. macerans* cannot grow in MacConkey broth; none of them is believed to be capable of growth in Teepol broth. False positive results, therefore, depend mainly on the relative frequency of occurrence of chlorine-resistant spores of *Cl. perfringens*, *B. polymyxa*, and *B. macerans* in the water samples. When chlorination is applied, sufficient to kill all coliform organisms, as in water treatment plants, the number of false positive reactions caused by chlorine-resistant spores would probably be increased as their presence would not be masked by positive reactions due to growth of coliform organisms.

Four laboratories examined a few samples of normally treated works-chlorinated waters containing no coliform organisms. All results were negative in 24 hr. The 48 hr. results are given in Table 5. This shows Gray's medium gave a large number

of false positive reactions compared with MacConkey broth, and Teepol broth gave none at all.

The third edition of Report 71 recommends that all MacConkey broth tubes showing acid and an amount of gas sufficient or more than sufficient to fill the concavity at the top of the Durham tube should be regarded as 'presumptive positives'. A lesser volume of gas may be disregarded unless visible gas appears in the liquid when the tube is lightly tapped. This means A 1 G 3 or possibly A 1 G 2 with the present notation. Since G 2 or G 3 is usually accompanied by at least A 2, it would be worth considering how many false positive results would be avoided by ignoring all reactions less than A 2 G 2 and how many coliform organisms would be missed. These results for all samples are summarized in Table 6.

Table 5. *False positive results given by samples of water containing no coliform organisms*

Number of tubes showing false positive reactions in 48 hr.*

A 1 G 1			A 2 G 2			
Oxoid MacConkey	Teepol Broth	Gray Glutamate	Oxoid MacConkey	Teepol Broth	Gray Glutamate	Total Tubes
7	0	30	2	0	26	176

* No false positive reactions were observed in 24 hr.

Table 6. *Effect of ignoring results less than A 2 G 2*

	Oxoid MacConkey broth		Teepol broth		Gray's medium	
	No. of tubes	% of positive results	No. of tubes	% of positive results	No. of tubes	% of positive results
Reduction in false positive results	68	2·7	34	1·3	72	2·5
Reduction in true coliform results	62	2·4	60	2·3	64	2·3

This table shows that some false positive results would be eliminated, but this would be offset by an approximately equivalent loss of true coliform results. The table also shows that there would be a small loss of true coliform results if the recommendation in the current 3rd edition of Report 71 were used.

It is suggested, therefore, that A 1 G 1 at 18 and 24 hr. should be accepted in the presumptive test for coliform organisms with all of these media. This will give quick results with a negligible number of false positive reactions. At 48 hr. if excessive numbers of false positive reactions are obtained with A 1 G 1 then A 2 G 2 could be accepted as a minimum with no greater loss of true coliform results than in the present recommended method.

Coliform organisms

As stated earlier a special series of twelve samples containing moderate numbers of coliform organisms and very few *Esch. coli* was examined at one laboratory. This enabled the suitability of the media to be assessed for coliform organisms without interference from *Esch. coli*. These samples were from new mains which had failed to give negative results despite repeated flushing. Although the mains were chlorinated originally, the water was not considered to contain chlorine resistant organisms, as the persistence of coliform organisms under these circumstances is usually due to growth on accumulations of dirt or other extraneous material in the mains. These results were, therefore, included in the unchlorinated series in Table 4 but they have been abstracted and shown separately in Table 7.

This shows that in 48 hr. Gray's medium was as successful for coliform organisms other than *Esch. coli* as it was in the other series for mixtures of these organisms. Their growth in Gray's medium was slow compared with that in MacConkey broth and Teepol broth and most of them gave poor acid and gas production in all media. The few *Esch. coli* that were present grew more readily in Gray's medium. The number of false positive reactions was high in all media but lowest with Gray's medium.

Adjustment of pH

In a previous trial in 27 laboratories, Gray's medium freshly prepared in his laboratory was found to give positive results equal to or greater than MacConkey broth even in 18 hr. (Gray, 1964), whereas in the current series growth was slower in Gray's medium. He suggested that this might be due to a difference in pH. Folpmers (1948) originally used media at pH 6·0 to inhibit growth of sporing bacilli, Gray (1959) found that pH 6·7 was the optimum and both Gray (1949) and Windle Taylor (1961–62) showed that less satisfactory results were obtained at pH 7·5. Gray obtained a pH 6·7 in the finished medium by adjustment of the pH to 6·8 before autoclaving. The pH of the Oxoid medium before autoclaving was 7·3 and observations at the different laboratories showed that final pH varied from 6·3 to 7·0. Various autoclaving techniques were used from 115° C. for 10 min. up to 121° C. for 15 min. and various shaped bottles and tubes either with screw caps, Oxoid caps or cotton wool plugs were used. All of these factors together with variations in sizes and types of autoclaves and hence rates of heating and cooling may effect the final pH. Gray, therefore, suggested that comparison should be made between Oxoid Gray's medium unadjusted and adjusted to pH 6·8 before autoclaving. A small number of samples was examined in this way and the results are summarized in Table 8. The final pH obtained after autoclaving these adjusted media varied from 6·1 to 6·7.

The results in Table 8 suggest that more *Esch. coli* were isolated and that coliform organisms and *Esch. coli* grew more rapidly in the pH adjusted medium. More acid and gas was also produced in the early stages. None of the differences in this table are however significant.

Table 7. *Comparison of MacConkey broth, Teepol broth, and Gray's glutamate medium on a series of new mains*

		% of all +ve reactions. False positive results			Number of tubes yielding								
					False positive results			Coliform organisms			*Esch. coli*		
		18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
A 1 G 1 or more	Oxoid MacConkey	0	6·7	27·1	0	4	16	22	43	59	1	2	2
	Teepol	0	5·5	23·6	0	3	13	29	41	55	1	1	1
	Gray glutamate	0	0	15·2	0	0	10	6	31	66	3	4	4
A 2 G 2 or more	Oxoid MacConkey	0	0	4·5	0	0	2	1	9	44	0	1	1
	Teepol	0	0	18·4	0	0	9	4	14	49	0	1	1
	Gray glutamate	0	0	1·9	0	0	1	0	4	52	0	2	4

Total no. of tubes examined 121.

Table 8. *Comparison of Gray's glutamate with pH adjusted and unadjusted*

	pH before auto-claving	Number of tubes yielding								
		False positive results			Coliform organisms			*Esch coli.*		
		18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.	18 hr.	24 hr.	48 hr.
A 1 G 1 or more	7·3	0	1	10	94	143	171	81	119	125
	6·8	4	4	12	99	149	174	87	128	133
A 2 G 2 or more	7·3	0	0	4	56	121	167	54	111	124
	6·8	0	0	3	69	135	172	66	118	133

Total no. of tubes examined 300.

Chlorinated samples

One outstanding peculiarity of Gray's medium indicated by these trials was its apparent superior ability to initiate growth of organisms, especially *Esch. coli*, which had been exposed to chlorine. One laboratory studied this phenomenon further by applying the marginal chloramine treatment described earlier to suspensions of *Esch. coli* in water. The resulting chlorinated suspensions were then added to MacConkey broth, lactose broth and Gray's medium.

When suspensions initially containing 500 to 5000 *Esch. coli* per 100 ml. were used, counts between 10 and 250 per 100 ml. after chlorination were regularly obtained with Gray's medium but no growth or only occasional growth was observed in tubes of MacConkey broth, or even in lactose broth which contains no inhibitory substances. In other words some of these organisms were viable in Gray's medium but not in ordinary nutrient media. It is easy to assume that these organisms are chlorine-damaged and the results suggest selective enzyme damage, but addition of some of the main ingredients of Gray's medium to MacConkey broth, singly or together, did not permit initiation of growth of these organisms. Conversely, omission of the supplementary growth factors from Gray's medium singly or collectively did not affect its ability to initiate growth of these organisms.

It has been recognized, however, that young bacterial cells are generally more sensitive to disinfectants or any other lethal agent, than older cells, and, furthermore, that the cells in a pure culture are not all of the same age (Walters, 1965; 1967). It follows, therefore, that marginal chlorination may merely be selecting the older cells. Some of these older cells may also be in a resting stage or at least have a prolonged generation time (Quesnel, 1963). This would account for the slow growth obtained in the trials.

DISCUSSION

These trials have indicated that Gray's medium is a satisfactory alternative to MacConkey broth for the detection of coliform organisms in water. It should not, however, be regarded as a second-best substitute. It is superior because of its ability to yield greater numbers of organisms, particularly of chlorinated or attenuated *Esch. coli.*

It could be argued that ability to recover damaged organisms is not essential in a medium; on the other hand, we do not know whether these organisms are chlorine-damaged or whether they are just chlorine-resistant nor whether they are viable in the body. Their presence certainly indicates marginal treatment. In view of the known resistance to chlorination of some viruses and of *Pseudomonas aeruginosa* the ability to isolate coliform organisms which survive marginal chlorination would provide an added safety factor in water treatment.

The original purpose in devising a chemically defined medium was that it should be standard in composition in all laboratories. It is a pity, therefore, that differences in pH occur so readily. This could be overcome at each laboratory by adjustment of the pH before autoclaving so that the medium for use has a final pH of 6·7.

The initial pH required should be determined in each laboratory, as it will probably vary according to the sterilizing equipment available and its method of use and the nature of the media containers and their methods of closure.

Had Gray originally used a mineral composition that avoided precipitation, confusion would not have arisen subsequently in attempts to avoid it. For samples of hard water addition of calcium and magnesium is unlikely to be necessary.

The adoption of Gray's medium for the bacteriological examination of water should not preclude the search for still further improvements. In view, however, of its superiority to other glutamate media, it is recommended that confusion should be avoided by rejecting the other glutamate media with which it has been compared.

Teepol broth can also be recommended as a satisfactory alternative to MacConkey broth but it will not give the same recovery of chlorinated organisms as Gray's medium.

These experiments were carried out with an Oxoid dehydrated version of Gray's medium which differed somewhat in mineral composition from the original. Further experiments are being carried out using a dehydrated medium which attempts to reproduce the mineral composition finally achieved by Gray (1964).

SUMMARY

Oxoid dehydrated MacConkey broth was compared with laboratory prepared MacConkey broth, Teepol broth and three modifications of glutamic acid media, by participants in eleven laboratories. A variety of chlorinated and unchlorinated water samples were used. The Oxoid MacConkey broth was shown to be a satisfactory standard for comparison. Teepol broth was found to be a satisfactory alternative to MacConkey broth. Gray's improved glutamate medium was shown to be the best of the glutamate media and it gave results superior in most respects to MacConkey broth. In particular more *Escherichia coli* were obtained as well as more coliform organisms. Organisms surviving marginal chlorination were recovered more readily. Growth was sometimes slower than in MacConkey broth but this could possibly be improved by closer attention to pH adjustment and mineral composition.

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