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Comparison of selenite F, Muller-Kauffmann tetrathionate and Rappaport's medium for the isolation of salmonellas from sewage-polluted natural water using a pre-enrichment technique

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SUMMARY

Three enrichment broths, selenite F, Muller–Kauffmann tetrathionate and Rappaport, were examined for their efficiency in salmonella isolation. The three media, prepared from single ingredients in the laboratory, were compared with their commercial equivalents. Laboratory-prepared media were more efficient for isolating salmonellas from sewage-polluted natural water samples. A pre-enrichment stage using buffered peptone water was employed throughout the investigation. The size of inoculum from the pre-enrichment medium was relevant to successful salmonella isolation. Inocula studied were 1 ml and one loopful (3 mm diameter loop). The smaller inoculum gave better results with Rappaport, the larger with selenite and tetrathionate. Using the optimal inocula, Rappaport was the most efficient enrichment broth of the three fluid media in this study.

INTRODUCTION

Many microbiologists would agree that fluid media based on sodium hydrogen selenite, sodium (or potassium) tetrathionate and a combination of magnesium chloride and malachite green provide efficient enrichment broths for the isolation of salmonellas. Historically, if we accept that malachite green is an important selective agent in the combination with magnesium chloride, then these media developed from the investigations of Klett (1900) and Guth (1916), Muller (1923) and Loeffler (1903, 1906). Further development may be credited to Leifson (1936), Kauffmann (1930, 1935) and Rappaport, Konforti & Navon (1956), and three widely used enrichment broths called selenite F, Muller–Kauffmann tetrathionate and Rappaport's medium evolved. These are the descriptive names we shall use in this study. Vassiliadis (1968) reviewed the literature on Rappaport's medium and Vassiliadis *et al*, (1970) modified the original formula by decreasing the amount of malachite green, Some comparisons of Rappaport's enrichment broth with

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selenite and tetrathionate have commended the malachite green medium (Collard & Unwin, 1958; Hooper & Jenkins, 1965). Other reports have not been so favourable (Sen, 1964). In 1957, in Cardiff, we made some unpublished comparisons between selenite, tetrathionate and malachite green magnesium chloride broth and corresponded with Rappaport on the possibility of his medium being modified for use at incubation temperatures above 37 °C. These elevated temperature studies were unsuccessful. The encouraging use of Rappaport's broth in Australia (Iveson, Kovacs & Laurie, 1964; Iveson & Kovacs, 1967), in Hong Kong (Chau & Huang 1971) and in Greece (Vassiliadis *et al.* 1972) prompted us to re-examine this medium and compare its efficiency with selenite and Muller–Kauffmann tetrathionate. The recording by several authors that optimum use of Rappaport's medium is dependent on the size of inoculum introduced led us to examine the effect of using two widely differing salmonella-containing inocula in the three enrichment broths.

Most papers on malachite green magnesium chloride broth have recorded results obtained by direct enrichment or secondary enrichment but Vassiliadis *et al.* (1972, 1976) found that Rappaport's medium could be successfully used after pre-enrichment in broth and buffered peptone water. As evidence is accumulating in food and water bacteriology that a pre-enrichment technique increases the number of salmonella isolations (Edel & Kampelmacher, 1973; Harvey & Price, 1977), we have followed this practice in the current study.

Some comparisons of selenite, tetrathionate and Rappaport's media have contrasted magnesium chloride malachite green broth prepared from ingredients compounded in the laboratory with other media purchased from commercial firms. In the present investigation we have preferred to prepare *all* three media from ingredients put together in our media room. A preliminary study has been made, however, comparing each of the three media under test with its nearest commercial counterpart. Media purchased ready-made from outside the laboratory have infiltrated so widely into microbiology that it is necessary occasionally to test their efficiency against each other (Read & Reyes, 1968) and against the same media made from their single constituents (Harvey, Price & Crone, 1975). Commercial media have been said to be more uniform in performance than the laboratory prepared product (Stokes 1978). This is contrary to our experience with some gram-negative enteric pathogens when small inocula are used (Harvey *et al.* 1975; Price, 1976).

Finally, we have examined the effect of subculture timing on positive results obtained with all enrichment broths. Only two times have been used: 24 and 48 h, although some workers have employed four subculture times between 24 and 96 h with good effect (Grunnet 1975). We aimed to complete a batch of tests within the working week. This allowed only two subcultures to be made.

MATERIALS AND METHODS

The purpose of the current study was to devise a test procedure which would probably reveal differences between the media examined. In a previous paper we

compared laboratory-prepared and commercial media using two different types of inoculum. One was a drop ($\frac{1}{50}$ ml) of a salmonella broth culture, the other was a a suitable volume of sewage-polluted natural water. The naturally contaminated inoculum allowed differences to be revealed between enrichment broths which were not evident when the artificially contaminated inoculum was used (Harvey *et al.* 1975). In the present trial, therefore, we employed a naturally contaminated salmonella inoculum. By trial and error, we found 25 ml of the river Taff collected on Mondays by one of the authors and brought immediately to the laboratory was suitable. The sample collected was sufficiently large to put up ten identical tests of 25 ml each week. The sampling point was at Pontypridd. The MPN of faecal coliforms at this stretch of the river is of the order of $4 \times 10^5/100$ ml (Smith, 1970). The trial period was January–December 1978, so that sampling was performed at all seasons of the year.

Selenite F broth (Leifson, 1936; Hobbs & Allison, 1954) was at one time the only salmonella enrichment medium used routinely in our laboratory. It is still used excusively for clinical diagnosis. It is prepared to the directions of Harvey & Price (1974) and is sterilized by Seitz filtration. This method of sterilization was originally suggested both by Leifson (1936) and by Hobbs & Allison (1945) as an alternative to heat sterilization. It has been used since 1945 in our laboratory. The final medium is crystal clear with no trace of red deposit of selenium which is sometimes found in heat-sterilized selenite F. Selenite broth sterilized by heat has been shown to inhibit multiplication of test organism *Shigella sonnei* (Price, 1976). In contrast, Seitz-filtered selenite broth permits growth of small inocula of this bacillus (1–10 organisms) in 24 h at 37 °C.

Muller–Kauffmann–tetrathionate broth was prepared in accordance with the International Standards Organization document ISO 3565 (Anon, 1975). This formula had been found to be somewhat more selective in an international trial than that previously used in Cardiff (van Schothorst *et al.* 1978; Harvey & Price, 1974).

Rappaport's medium was made to the formula of Vassiliadis *et al.* (1970) who decreased the amount of malachite green in the enrichment broth. This minor change resulted in better growth of salmonellas. Our own experience of reducing the concentration of a selective agent in an enrichment broth has enabled positive salmonella isolations to be obtained at an early subculture time. The time of first positive subculture is related to the number of salmonellas in the inoculum (MacCoy, 1962) and to the concentration of selective agent in some tetrathionate media (Harvey & Price, 1979). By analogy, the change in Rappaport's original formula, therefore, seemed sensible as considerable saving of media can be obtained if early subculture is successful.

Buffered peptone water (van Schothorst & van Leusden, 1972) was prepared according to the International Standardization document (Anon, 1975).

Brilliant green MacConkey agar (Harvey, 1956), which was used exclusively for plating in the trial, was made to the instruction of Harvey & Price (1974). This is a medium which allows growth of a wide range of salmonella serotypes (Chau & Leung, 1978).

In the preliminary tests of commercial and laboratory-made enrichment media, 25 ml of river water was added to 25 ml of double-strength buffered peptone water. This was incubated at 37 °C for 18 hours. Subcultures were then made, using an inoculum of 1 ml, to selenite F, Muller–Kauffmann tetrathionate and Rappaport's broth prepared as above and to the commercial equivalents of these media (Oxoid CM 399, Oxoid CM 343 and Gibco T4015). The pairs of media were incubated at 43 °C in the case of selenite and tetrathionate (Harvey & Thomson, 1953; Edel & Kampelmacher, 1969) and at 37 °C for the paired Rappaport broths. Subcultures were made after 24 and 48 h incubation to brilliant green MacConkey agar. Selective agars were incubated at 37 °C for 24 h and examined for salmonella colonies.

The main trial, in this study, involved evaluating the relative efficiency of the three media: selenite, tetrathionate and Rappaport prepared from ingredients compounded in the laboratory. It followed the same pattern as the preliminary trial already described. Twenty five milli-litres of river water were added to 25 ml of double-strength buffered peptone water. This was incubated at 37 °C for 18 h. Two widely differing inocula were taken from the incubated buffered peptone water. The smaller inoculum (one loopful using a 3 mm diameter loop) was introduced into 10 ml of selenite F, and the larger inoculum (1 ml) was introduced into an identical volume of the same medium. In a similar manner these two inocula were added to paired 10 ml volumes of Muller–Kauffmann tetrathionate broth and Rappaport's broth. Six enrichment cultures were therefore put up from the single-buffered peptone water culture. Ten sets of 25 ml of river water were cultured from each large sample of river water each Monday so that 60 enrichment broths were examined during the working week. Selenite and tetrathionate broths were incubated at 43 °C. Rappaport broths were incubated at 37 °C. Subcultures were made after 24 and 48 h incubation to brilliant green MacConkey agar. Selective agars were incubated at 37 °C for 24 hours and examined for Salmonella colonies.

RESULTS

The results of this media trial are set out in tables 1–5. Tables 1 and 2 record non-concordant data only as these are all that is necessary to make their point. Laboratory-prepared media are, in this study, more efficient than equivalent commercial media and the volume of the inoculum from the pre-enrichment medium is relevant to the efficient use of all three media tested. The 1 ml inoculum of buffered peptone water culture gave better isolation rates with selenite and tetrathionate while the loopful was more efficient with Rappaport. Taking these facts into consideration, table 3 examines the three media as prepared in the laboratory from single ingredients using appropriate inocula. In this series, Rappaport gave significantly better isolation rates than selenite and Muller–Kauffmann tetrathionate. Table 4 examines the relation between time of subculture and salmonella isolation. As indicated, two subcultures only were possible to finish tests within the working week. Rappaport obtained 90·7 % of possible positive results at the 24 h subculture and 93·1 using two subcultures. Table 5 arranges

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Table 1. *Comparison of laboratory prepared and commercial media*

Enrichment medium	Lab medium positive Commercial medium negative	Lab medium negative Commercial medium positive	χ^2	P
Selenite	77	3	66·6	$<0{\cdot}01$
Muller–Kauffmann tetrathionate	38	3	28·2	$<0{\cdot}01$
Rappaport	39	0	37·0	$<0{\cdot}01$

results in frequency distribution form. Equivalent salmonella MPN are recorded in this table.

DISCUSSION

The current investigation records findings with three enrichment media relevant to isolation of salmonellas from naturally contaminated water containing small numbers of these organisms (table 5: 0·42 – 9·2 + salmonellas/100 ml). One should not assume that all salmonella-containing material will give similar results with selenite, tetrathionate and Rappaport. This problem is now being studied with poultry samples. In a previous paper we noted that direct inoculation of poultry offal into commercial Muller–Kauffmann tetrathionate (Oxoid CM 343) did not give the same results as contaminated water inocula in the same medium (Harvey *et al.* 1975). We suggested that soluble substances diffusing from the poultry advantageously altered the efficiency of the commercial medium. With a pre-enrichment technique, as used in the present study, this would be less likely to happen.

Commercial media function well for many types of salmonella isolation problems. In faecal samples in intestinal diseases, numbers of salmonellas may range from 10^4 to 5 to 50 $\times 10^7$ (Thomson, 1955). Such specimens usually give a good growth of pathogens on media purchased from commercial firms. We have also found that samples of minced meat examined by a pre-enrichment technique are efficiently cultured using commercial selective and enrichment media (van Schothorst *et al.* 1978). In the present investigation, however, laboratory-prepared media give significantly better results (table 1). For quantitative estimations of salmonellas in sewage-polluted natural water, commercial enrichment broths are less efficient than the laboratory-prepared equivalent. We have already indicated this for direct enrichment; the current study using pre-enrichment in buffered peptone water extends our earlier findings (Harvey *et al.* 1975).

Table 2 presents data on the effect of varying the inoculum from pre-enrichment to enrichment medium. Jameson (1963) drew attention to the importance of the ratio of the volume of the inoculum from the primary enrichment medium to the volume of the secondary enrichment medium. It is obviously of importance also in pre-enrichment techniques (table 2). It should be noted that the large inoculum (1 ml) favoured results obtained from selenite and tetrathionate, while the small

Table 2. *Effect of varying inoculum from pre-enrichment medium to enrichment media* (3 *mm diameter loopful* versus 1 *ml*)

Enrichment medium	1 loopful from pre-enrichment medium to enrichment medium: positive 1 ml inoculum negative	1 loopful from pre-enrichment medium to enrichment medium: negative 1 ml inoculum positive	χ^2	P
Selenite F	14	48	17·6	<0·01
Muller–Kauffmann tetrathionate	16	50	16·5	<0·01
Rappaport	99	8	75·7	<0·01

Table 3. *Relative efficiency of selenite, tetrathionate and Rappaport*

Medium positive or negative	Inoculum		χ^2	P
Muller–Kauffmann tetrathionate positive Selenite F positive	168	1 ml for tetrathionate		
Muller–Kauffmann tetrathionate positive Selenite F negative	27	1 ml for selenite F	2·1	>0·1
Muller–Kauffman tetrathionate negative Selenite F positive	40			
MK tetrathionate positive Rappaport positive	185	1 ml for tetrathionate		
MK tetrathionate positive Rappaport negative	10	1 loopful for Rappaport	21·0	<0·01
MK tetrathionate negative Rappaport positive	45			
Selenite F positive Rappaport positive	195	1 ml for selenite F		
Selenite F positive Rappaport negative	13	1 loopful for Rappaport	9·2	<0·01
Selenite F negative Rappaport positive	35			
Total tests = 350	Total positives with combined results of three media = 247			

inoculum (1 loopful) was best with Rappaport. A similar conclusion was recorded by Collard & Unwin (1958).

Table 3 examines the three media at their optimum as revealed by tables 1 and 2. Thus, laboratory-prepared enrichment broths only were studied and inocula adjusted to give the best results with each medium tested. Rappaport's medium gave significantly better results than selenite or tetrathionate. This contrasts with Grunnet's (1975) findings but his comparison used 42 °C as incubation temperature without a pre-enrichment stage. Recently a modification of Rappaport's medium has been described for use at 43 °C (Vassiliadis *et al.* 1978) but we have preferred

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Table 4. *Timing of subculture from enrichment media producing positive negative results*

Subculture positive or negative		Selenite F (1 ml inoculum)	Tetrathionate (1 ml inoculum)	Rappaport (loopful inoculum)
24 h+	48 h+	175	138	211
24 h+	48 h−	4	11	13
24 h−	48 h+	29	46	6
24 h−	48 h−	142	155	120
24 h+		179 (72·5)	149 (60·3)	224 (90·7)
48 h+		203 (82·6)	184 (74·5)	217 (87·9)
24 h and 48 h combined		208 (84·2)	195 (78·9)	230 (93·1)
Total possible positives by all media combined		247	247	247

Figures in parenthesis are percentages of total possible positives combining results from all three media.

Table 5. *Positive salmonella isolations: frequency distribution of score out of ten with different media and inocula*

Equivalent MPN per 100 ml...	0·42	0·89	1·43	2·04	2·77	3.66	4·8	6·44	9·2	9·2+	Total samples positive
No. of 25 ml samples out of ten positive...	1	2	3	4	5	6	7	8	9	10	
Medium and Inoculum											
Selenite loop	4	2	1	2	4	3	4	4	2	4	175
Selenite 1 ml	1	3	2	4	5	5	3	1	5	5	208
Tetrathionate loop	2	8	2	3	5	1	3	2	4	2	160
Tetrathionate 1 ml	3	4	3	2	2	7	3	1	4	5	195
Rappaport loop	2	2	3	2	1	5	5	5	3	7	230
Rappaport 1 ml	5	3	5	3	4	0	4	4	1	1	137

Greater MPN with selenite 1 ml than with Rappaport loop = 5
Greater MPN with Rappaport loop than with selenite 1 ml = 17
Greater MPN with tetrathionate 1 ml than Rappaport loop = 4
Greater MPN with Rappaport loop than with tetrathionate 1 ml = 18
Relative efficiency of methods: Rappaport loop > Selenite 1 ml > Tetrathionate 1 ml > Selenite loop > Tetrathionate loop > Rappaport 1 ml
The MPN values were calculated from tables recorded by McCoy (1962)

to study a Rappaport's broth suitable for salmonella enrichment at 37 °C. Not every laboratory wishes to duplicate incubation temperatures and 37 °C is still convenient for most microbiological techniques.

Table 4 records the success of subcultures from the three enrichment media made at 24 and 48 h. The diagnosis of 90·7 % of the total possible positive results at the 24 h subcultures with Rappaport's medium is worth emphasizing. This figure compares with 60·3 % for tetrathionate and 72·5 % for selenite. Speed of diagnosis is therefore an attribute of Rappaport's broth. Other properties favour Rappaport. It is a medium of good keeping quality and is cheap to prepare. It allows multiplication of a wide range of salmonella serotypes (Vassililiadis *et al.* 1974) but is not optimum for isolating *S. typhi* (Rappaport *et al.* 1956) or *S. dublin* (Harvey &

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Price, 1975). The combination of enrichment media, Rappaport with selenite or strontium selenite, has much to commend it in countries where *S. choleraesuis*, *S. typhi* and a wide range of other salmonella serotypes are found (Chau & Huang, 1971).

Table 5 arranges data from the six tests carried out in terms of frequency distributions of the number of salmonella isolations out of 10-samples of 25 ml of water. The most efficient technique used Rappaport with a loopful inoculum. The least successful was Rappaport with 1 ml inoculum. Table 5 also records the greater MPN salmonella counts obtained with Rappaport in comparison with selenite and tetrathionate. For quantitative salmonella estimations on sewage-polluted natural water in this series Rappaport was the most efficient enrichment medium.

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