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A comparison of two modifications of Rappaport's enrichment medium (R25 and RV) for the isolation of salmonellas from sewage polluted natural water

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SUMMARY

The development of Rappaport's enrichment medium and elevated temperature incubation as methods of salmonella isolation is traced. The recent recording of a merger of the two techniques by means of Rappaport–Vassiliadis medium is noted (RV medium).

In Cardiff, we have found an earlier modification of Rappaport's enrichment (R25) by Vassiliadis to be efficient in salmonella recovery from environment samples. The current study compares the two media using sewage polluted natural water as test material. Under the conditions of experiment, R25 was more successful in salmonella isolation than RV, although the latter medium inhibited competitive organisms better.

R25 is a convenient enrichment broth for routine use. In combination with pre-enrichment it allows the use of a loop for subculture rather than a pipette. This increases safe manipulation. It also produces a high proportion of positive isolations at the 24 h subculture time in contrast to other enrichment broths. For these reasons it forms an integral part of salmonella isolation methodology in our laboratory.

INTRODUCTION

Rappaport, Konforti & Navon (1956) in Israel described a new enrichment medium for salmonellas based on two selective ingredients, magnesium chloride and malachite green. Rappaport & Konforti (1959) stressed the importance of using Bacto Tryptone in the medium, or one of the Bacto supplements to ensure satisfactory isolation of *Salmonella paratyphi A*. This serotype is frequently encountered in the Middle East. Some strains show minimal gas production in sugars (Dick, 1946). The magnesium chloride/malachite green medium was given qualified approval by Collard & Unwin (1958) and Hooper & Jenkins (1965), but Sen (1964) was disappointed in its performance. Iveson, Kovacs & Laurie (1964) in Australia, found the medium efficient for isolating salmonellas from contaminated

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coconut. Chau & Huang (1971) in Hong Kong, reported favourably on Rappaport's medium in a study of faecal samples of hospital patients who were not suffering from diarrhoea or enteric fever. The enrichment medium has, therefore, received approval from laboratories in several parts of the world. All these studies used Rappaport's original concentration of malachite green in the formula. The incubation temperature in every case was 37 °C.

The first modification of Rappaport's medium was described by Vassiliadis *et al.* (1970) in their study of Greek abattoirs. By reducing the concentration of malachite green slightly in the enrichment broth, they obtained a better growth of salmonellas without impairing selectivity. We adopted this modification (R25 medium) in comparative trials of the common salmonella enrichment broths (Harvey, Price & Xirouchaki, 1979; Harvey & Price, 1981). In our hands, the R25 modification was the most efficient of the enrichment media studied.

The device of elevated temperature incubation – using temperatures above 37 °C to incubate salmonella enrichment media and thereby increase selectivity is of long standing. It was first described by Rodet (1889) who used 44·5 °C. Vincent (1890) had little success with the technique but by lowering the incubation temperature to 42 °C recorded a method by which *S. typhi* was isolated from river water. Harvey & Thomson (1953) noted that if selenite F broth (Leifson, 1936) was incubated at 43 °C, an increase in salmonella isolations was obtained and selectivity was enhanced. Harvey & Phillips (1961) used selenite F at 43 °C to good effect in an environmental survey of bakehouses and abattoirs for salmonellas and Harvey & Price (1968) reviewed their evidence, collected over 14 years, for adopting 43 °C as optimum incubation temperature for selenite F broth used for salmonella recovery from environmental samples.

Other microbiologists have confirmed the advantages of elevated temperature enrichment with different media. Spino (1966) reduced the temperature to 41·5 °C. He used SBG-sulfa enrichment and tetrathionate (formula unstated). A standardized technique for salmonella isolation was based on the discovery by Edel & Kampelmacher (1969) that Muller–Kauffmann tetrathionate broth (tetrathionate concentration 0·018 M – Knox, Gell & Pollock, 1943) was efficiently used at 43 °C.

By reducing the malachite green concentration in Rappaport's enrichment broth by more than half, Vassiliadis *et al.* (1976) developed a medium capable of successful salmonella recovery at 43 °C incubation. The medium was, at first, called R10 and later it was given the name Rappaport–Vassiliadis enrichment (RV) medium. Recently, Vassiliadis (1983) reviewed his experience of RV medium in comparative trials against other techniques. In Cardiff, as we have said, we had been impressed by the efficiency of the original modification of Rappaport (R25). It was logical, therefore, to compare the two modifications R25 and RV. The results of this study are recorded in the current paper.

MATERIALS AND METHODS

The media used were buffered peptone water (van Schothorst & van Leusden, 1972), R25 and R10 modifications of Rappaport's enrichment medium (Vassiliadis *et al.* 1970; Vassiliadis *et al.* 1976), brilliant green MacConkey agar (Harvey, 1956) and, in the later stages of the study, deoxycholate citrate agar (Leifson, 1935;

Hynes, 1942). The test material, as in an earlier investigation (Harvey, Price & Crone, 1975) was 25 ml of sewage polluted natural water.

The general technique was to pre-enrich the water sample in buffered peptone water, subculture to the two enrichment media and finally to subculture from the enrichment broths to a selective agar for differentiation and identification of salmonella colonies.

In an earlier study, we found that an appropriate inoculum from the pre-enrichment culture to an enrichment medium incubated at 43 °C was greater in volume than that suitable for the same enrichment medium incubated at 37 °C (Harvey & Price, 1982*a*). As the RV medium, in the current investigation, was to be incubated at 43 °C, it was necessary to determine the size of inoculum from the peptone water culture that would give the maximum salmonella recovery from enrichment. Twenty five millilitres double strength buffered peptone water was inoculated with 25 ml of sewage polluted natural water and incubated at 37 °C for 18–24 h – the pre-enrichment culture. Five test tubes were taken each containing 10 ml of RV medium. The first tube was seeded with one loopful (0·005 ml) pre-enrichment culture. The second and third tubes were seeded with one drop and five drops respectively (0·02 and 0·1 ml) and the fourth and fifth tubes with 0·5 and 1·0 ml. A graduated loop and graduated capillary pipettes were used for loop and drop volume measurements. The tubes were incubated at 43 °C for 48 h and were subcultured at 24 and 48 h to brilliant green MacConkey agar. Selective agars were incubated at 37 °C for 24 h and salmonella colonies were identified on the plates. The whole process was repeated with 149 further samples of sewage polluted natural water. From these investigations the most suitable inoculum for the RV medium was determined as 0·5 ml of peptone water culture. We had already found the appropriate inoculum for the R25 medium to be one loopful (0·005 ml) of the pre-enrichment culture in a similar manner (Harvey & Price, 1980). Using these two values, we were able to proceed with the comparative trial of R25 and RV media.

Twenty five millilitres of sewage polluted water were added to 25 ml of double strength buffered peptone water and incubated at 37 °C for 18–24 h. Two test tubes were taken, one containing 10 ml of R25 medium, the other 10 ml of RV medium. From the peptone water culture, one loopful (0·005 ml) was inoculated into the R25 tube while 0·5 ml was introduced into the RV tube. The R25 enrichment was incubated at 37 °C for 48 h and the RV enrichment at 43 °C for 48 h. Both media were subcultured at 24 and 48 h to brilliant green MacConkey agar. The selective agars were incubated at 37 °C for 24 h and were examined for salmonella colonies. The process was repeated with other specimens of polluted water. In all, 490 samples were examined.

Near the end of the trial, deoxycholate citrate agar was used as additional plating medium in parallel with brillant green MacConkey agar.

RESULTS

The results of the investigation are presented in Tables 1–4. Table 1 records the relationship between the volume of inoculum seeded from the pre-enrichment culture to the RV medium and the number of salmonella isolations obtained. In

Table 1. *Salmonella isolation from RV medium with different inoculum volumes from the pre-enrichment culture*

Inoculum volume (ml)	0·005	0·02	0·1	0·5	1·0
Number of salmonellae isolations	43	45	50	67	54
Number of 'sterile' plates observed	68	60	35	15	5
Total samples			150		

Table 2. *Salmonella isolations from sewage polluted natural water using R25 and RV media*

Medium positive or negative	Number of salmonella isolations
R25 positive RV positive	228
E25 positive RV negative	59
R25 negative RV positive	26
R25 negative RV negative	177
Total samples positive with both media	313
Total samples examined	490

Table 3. *Timing of positive subculture from R25 and RV enrichment media*

Subculture positive or negative	R25	RV
24 and 48 h positive	233	205
24 positive 48 h negative	39	19
24 negative 48 h positive	15	33
24 negative 48 h negative	203	233
Total 24 h subcultures positive	272 (87)	224 (72)
Total 48 h subcultures positive	248 (79)	238 (76)
Combined positive results using 24 and 48 h subcultures	287 (92)	257 (82)
Total samples positive with both media	313 (100)	
Total samples examined	490	

Figures in parentheses are percentages as integers.

Table 4. *Comparison of Brilliant Green MacConkey Agar (BG) and deoxycholate citrate agar (DC) as plating media*

Plating medium positive or negative	Enrichment medium and subculture time				
	R25/24 h	R25/48 h	RV/24 h	RV/48 h	Total
BG+ DC+	66	55	45	39	205
BG+ DC−	4	12	7	13	36
BG− DC+	0	0	2	0	2
BG− DC−	50	53	66	68	237
Total water samples		120			
Total paired platings					480

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this series, 0·5 ml of peptone water culture gave the best salmonella recovery and this value was taken as appropriate for the trial of RV medium against R25. We noted and recorded the number of selective agars in the study on which no apparent growth was visible. The number of these 'sterile' plates observed decreased with increasing inocula volume.

Table 2 presents the data of the R25/RV trial. The samples were paired and in this series R25 was the better medium (McNemar's χ^2 test: $P < 0{\cdot}01$).

Table 3 records isolations at 24 and 48 h subcultures from two enrichment media.

If the results recorded in Table 3 are examined in which a medium yielded a positive result either at 24 h or at 48 h but not at both subculture times, the pattern shown by the two enrichment broths is different. More isolations were made exclusively at 24 h with the R25 medium. With the RV enrichment the reverse pattern is noted. It is worth emphasizing that 87 % of the possible positive results were obtained with R25 at the 24 h subculture.

Table 4 compares salmonella isolations by brilliant green MacConkey and deoxycholate citrate agar. We had not made a formal comparison between these two selective agars since 1956 (Harvey, 1956). The present study provided a good opportunity to re-examine our earlier results. Taking the figures in the last column of Table 4 and applying McNemar's χ^2 test $P < 0{\cdot}01$. In this study, brilliant green MacConkey is the better medium. It is also a selective agar that does not require more than 24 h incubation, in contrast to deoxycholate citrate agar, to produce easily differentiated salmonella colonies.

DISCUSSION

Table 1 records a clear association between the size of inoculum, salmonella recovery and growth inhibition ('sterile' plates) with the RV medium incubated at 43 °C. A similar result was obtained with strontium chloride B enrichment. The latter was also incubated at 43 °C (Harvey & Price, 1982*a*). When two enrichment media are compared, they are usually treated in exactly the same way in a trial. With these two Rappaport media, this could not be done without biasing the comparison result. If the inoculation volume 0·005 ml was used for both media, the R25 enrichment would be favoured while if 0·5 ml were employed the RV medium would be at an advantage. The only way to achieve a valid comparison is to adjust inocula to what seems optimum for each medium, with the test material sewage polluted natural water. Other contaminated samples might give different results.

The use of a loop as inoculating instrument (0·005 ml inoculum) is convenient to British microbiological practice which does not approve mouth pipetting on safety grounds (Collins, 1974). The numbers of salmonellas per ml in a peptone water culture would be large and potentially infective. It must not be forgotten, however, that a pre-enrichment stage in the routine is needed to ensure that viable salmonellas capable of multiplying in enrichment media are present in such a small volume as 0·005 ml. A pre-enrichment stage, however, is not always successful (Siebeling, Neal & Granberry, 1975). Where large inocula, such as 80 g, are to be introduced into an enrichment medium as in an earlier paper on salmonellas in pig faeces, the R25 broth might not be so efficient as in this study. Both selenite F

and Muller–Kauffmann tetrathionate can accept large inocula and might be more suitable media (Harvey, Price & Morgan, 1977).

The effective inocula (0·005 and 0·5 ml) used with R25 and RV broths differ by a factor of 100. Salmonella organisms are being seeded to the RV medium incubated at 43 °C which are recovered at 37 °C with R25 but not at elevated temperature (Table 1). We may deduce that the salmonella population in the pre-enrichment culture is not homogeneous and may consist of cells capable of multiplying in buffered peptone water only, cells capable of multiplying in R25 only and cells able to grow both at 37 and 43 °C in R25 and RV respectively. This implies that the peptone water culture is merely an extension of the state of affairs existing in the original 25 ml water sample. Other microbiologists have drawn attention to failure to obtain satisfactory salmonella recovery at 43 °C from river water and sewage effluent samples (Burman, 1967; McCoy, 1962).

In the current study, R25 was more efficient than RV in salmonella recovery from sewage polluted water. These results, however, must not be projected without further experiment to cover other materials. Test samples can have a profound influence on outcome. In a study of Oxoid CM343 Muller–Kauffmann tetrathionate different findings were made with chicken giblets to those obtained with sewage polluted natural water (Harvey, Price & Crone, 1975). In a survey of salmonellas in symptomless pigs, selenite F was a better medium than Muller–Kauffmann tetrathionate with pig faeces. With lymphatic glands, the reverse was the case (Harvey, Price & Morgan, 1977).

RV medium is more selective than R25 as evidenced by its greater inhibition of competitive organisms and might be the better enrichment broth with other test materials. In a comparison of RV enrichment made with soya peptone and the ISO procedure with Oxoid CM343 (Anon, 1981), van Schothorst & Renaud (1983) obtained better results with the Rappaport's medium. The two media (R25 and RV) used in combination might be worth considering. This device is of long standing in microbiology. At one time, brilliant green peptone water was used in three different strengths for each sample to aid salmonella isolation.

In Table 3, we noted a difference in the salmonella recovery pattern in the two media. Salmonellas are isolated more often at the 24 h subculture with R25 than with RV. Early recovery of salmonellas is an advantage and has prompted several studies in the past (Dixon, 1961) and more recently (Price, 1983; van Schothorst & Renaud, 1983). Time of isolation of salmonellas from enrichment appears to be related to the numbers present in the original inoculum (McCoy, 1962) and also the concentration of the selective agent in the fluid medium (Harvey & Price, 1979). The later appearance of demonstrable salmonellas in RV broth could be correlated with its greater selectivity.

Table 4 confirmed our belief that brilliant green MacConkey agar was the best choice for plating from enrichment. Its use also assists rapid isolation of salmonellas as the selective agar gives good differentiation with 18–24 h incubation.

We have now tested the R25 enrichment against selenite F, Muller–Kauffmann tetrathionate, strontium chloride B and RV enrichment (Harvey, Price & Xirouchaki, 1979; Harvey & Price, 1981; 1982*a*). We have been impressed with its performance and have based much of our salmonella isolation technique in Cardiff on its use (Harvey & Price, 1982*b*)

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