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Multiple plating from enrichment media as an aid to salmonella isolation

By T. H. PRICE

Regional Public Health Laboratory, University Hospital of Wales, Heath Park, Cardiff, CF4 4XW

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SUMMARY

Faeces samples enriched for salmonellas in selenite F broth incubated at 43 °C were subcultured to brilliant green MacConkey agar after 6 h. Four platings were made. As a control technique, each sample was enriched in selenite F incubated at 37 °C for 24 h and subcultured once to brilliant green MacConkey agar. Of the specimens plated at 6 h, out of a total of 25 positive samples, 6 required more than a single subculture to reveal the presence of salmonellas. Twenty-four positive isolations were made from the selenite broths incubated at 37 °C for 24 h and subcultured once only. All faecal samples positive by enrichment were also positive by direct plating. Nine samples seeded by 6 h produced only 1–2 salmonella colonies per plate. Convalescent specimens are, therefore, unlikely to be efficiently diagnosed by early subculture even if it is combined with multiple plating.

River Taff samples, after pre-enrichment in buffered peptone water, were enriched in Rappaport's medium at 37 °C for 6 h. Subcultures were made to four plates of brilliant green MacConkey agar. A control subculture was made at 24 h to a single plate of brilliant green MacConkey. Although multiple subculture at 6 h improved salmonella isolation, the method was not as efficient as a single plating from the same enrichment culture at 24 h. There was no evidence that subculture at 6 h combined with multiple plating had a value either for faecal examination or for the isolation of salmonellas from sewage-polluted natural water.

Samples of other natural waters submitted routinely by Environmental Health Officers were pre-enriched in buffered peptone water before subculture to Rappaport's medium. The enrichment cultures were incubated at 37 °C for 24 h and duplicate platings were made to brilliant green MacConkey agar. By double plating at 24 h, a gain of 16 % in salmonella recovery was made. This compares favourably with the gain obtained by subculture from Rappaport after 24 and 48 h incubation. It also saves 24 h in examination time.

INTRODUCTION

Loeffler (1906) was one of the first microbiologists to record the association between duration of growth of an organism in a fluid medium and the number of subculture plates required for its recovery. He calculated that, for the isolation of *Salmonella typhi* from 100 ml of broth inoculated with 1–2 organisms, 25 plates

would be necessary after 10 h incubation at 37 °C. The plating inoculum used was 1/10 of a loopful. Under the same circumstances, only one plate would be needed after 16 h incubation. The effect of competing organisms did not enter into the calculations.

Drigalski & Conradi (1902), in a study on *S. typhi* isolation, drew attention to the advantages of quadruple plating for direct culture of clinical specimens. The success of this technique probably lay in obtaining satisfactory separation of colonies on the plate surface with optimal differentiation of pathogen from non-pathogen. The same reasoning was applied many years later to the recovery of *S. typhi* by Moore (1950) who plated a series of tenfold sewage dilutions to selective agars. Increased availability of selective agar surface for adequate colony separation may be obtained, either by increasing the number of plates used, or by increasing the size of the Petri dish employed (Edel & Kampelmacher, 1968).

Multiple plating can also compensate for a variable distribution of salmonellas throughout the sample. In some cases, homogenizing is more effective than multiple plating (Kröger, 1951). This technique is an integral part of salmonella isolation from certain foods.

Dixon (1961) attempted to shorten the time required for salmonella examination of faeces by selenite enrichment at 43 °C (Harvey & Thomson, 1953) combined with subculture to brilliant green MacConkey (Harvey, 1956) at 6 h. His results, however, compared unfavourably with plating from the same enrichment medium at 24 h. Harvey & Price (1962*a*) noted the value of quadruple plating from selenite broth incubated at 43 °C. The method appeared to be useful when scanty salmonellas were present in the broth at the subculture time selected (24 h). It was considered that the two techniques might be combined: multiple plating with 6 h subculture. This paper records an investigation of the method using selenite F and Rappaport's magnesium chloride malachite green broth as modified by Vassiliadis *et al.* (1970).

MATERIALS AND METHODS

Materials examined in this study were routine faecal specimens, samples collected by the author from the River Taff, and various natural water samples regularly submitted by environmental health officers. A few sewage effluents were included with the latter specimens.

The media used were buffered peptone water (Anon, 1975), selenite F broth (Leifson, 1936), magnesium chloride malachite green broth (Rappaport, Konforti & Navon, 1956; Vassiliadis *et al.* 1970) and brilliant green MacConkey agar (Harvey, 1956). Preparation of these media is described by Harvey & Price (1982*b*). All were compounded in the laboratory from individual ingredients.

Faecal samples were submitted as faecal swabs (a throat swab dipped in faeces and posted or delivered to the laboratory). These were agitated in 5 ml of peptone water to make a faecal suspension and coarse particles were allowed to settle. Three drops of the suspension (approximately $3 \times 0{\cdot}02$ ml) were inoculated into 10 ml of selenite F broth. This inoculation was duplicated. One of the selenite cultures was incubated at 43 °C for 6 h and then subcultured to four plates of brilliant green MacConkey agar. The second selenite broth was incubated at 37 °C for 24 h and

Table 1. *Salmonella isolation by multiple plating from enrichment media*

Test material...	Faeces	River water	Environmental samples
Enrichment medium...	Selenite	Rappaport	Rappaport
No. of samples positive at first plating from enrichment	19	11	32
Cumulative no. of samples positive with			
2 platings	22	14	38
3 platings	24	14	—
4 platings	25	20	—
Incubation time	6 h	6 h	24 h
Temperature of enrichment	43 °C	37 °C	37 °C
Percentage gain of multiple plating over single plating as an integer	24	45	16

subcultured to a single plate of brilliant green MacConkey agar. Selective agars were incubated at 37 °C for 24 h and examined for salmonella colonies.

Water was collected in a litre bottle from the River Taff at Pontypridd and taken immediately to the laboratory. There, ten separate 25 ml volumes were removed. One 25 ml quantity of water was added to 25 ml of double strength buffered peptone water and incubated at 37 °C for 18 h. A loopful of this culture (approximately 0·005 ml) was then introduced into 10 ml of magnesium malachite green enrichment broth which was incubated at 37 °C for 6 h. Subcultures were made to four plates of brilliant green MacConkey agar. Plates were incubated at 37 °C for 24 h and examined for salmonellas. The remaining nine 25 ml samples of river water were treated in exactly the same way. As a control, each of the ten Rappaport's enrichment broths was subcultured to a single plate of brilliant green MacConkey agar after 24 h incubation.

Samples submitted by environmental health officers were treated in a somewhat similar way, but the 6 h subculture was omitted and a plating at 24 h only was made from enrichment. A 25 ml volume of the specimen was added to 25 ml of double strength buffered peptone water and incubated at 37 °C for 18 h. A loopful of this culture (0·005 ml) was seeded to 10 ml of Rappaport's broth and incubated at 37 °C for 24 h. The enrichment medium was subcultured twice to whole plates of brilliant green MacConkey agar. The two selective agars were incubated at 37 °C for 24 h and then examined for salmonellae.

RESULTS

The results are recorded in Tables 1, 2 and 3 which are largely self-explanatory.

Table 1 presents the cumulative increase in the number of salmonella isolations obtained by multiple plating. Twenty-five faecal samples were positive for salmonellas at the 6 h subculture. Sixteen of these were positive on all 4 platings. In nine

Table 2. *Source of mixed environmental samples*

Sample	Total positive for salmonellas	Samples negative by single plating but positive by duplicate
Dock feeder	1	—
Effluent	10	1
Farm stream	1	—
River Ely	3	—
River Ogmore	9	2
River Rhymney	3	1
River Taff	4	—
River unspecified	1	1
Roath Park Lake	1	—
Sea water	5	1
Total	38	6

Table 3. *Comparison of salmonella isolation at 6 and 24 h subcultures*

	Faeces	River Taff Samples
Samples positive at 6 h (four platings)	25	20
Samples positive at 24 h (one plating)	24	76
Samples positive combining results obtained with both subcultures	27	78

With faecal specimens, the 6 and 24 h subcultures were made from separate enrichment broths, as each was incubated at a different temperature. With river water samples, the two subcultures were made from the same enrichment broth.

samples some negative plates, out of four per sample, were recorded. Of 36 plates from these specimens (4×9), 14 presented with salmonella colonies. The number of salmonella colonies per plate was noted. On 11 plates, only one colony was identified and on 3 plates only two colonies were found. Low salmonella colony identification is, therefore, associated with 6 h subculture from enrichment, which is to be expected.

Table 2 records the origin of the routine environmental samples with the results of duplicate plating from Rappaport's medium incubated at 37 °C for 24 h.

Table 3 compares salmonella isolation at 6 h with that obtained by orthodox subculture at 24 h. With the faecal specimens, no difference is evident, but with the river water samples, a 6 h plating on 4 plates obtained only 20 (26 %) of the possible positives. Three salmonella isolations were made at 6 h with faeces which were not found at 24 h. Two positive results were obtained with water specimens at 6 h, but not at 24 h.

DISCUSSION

It is not surprising that little difference in salmonella recovery was obtained at the 6 h and 24 h subcultures with faecal specimens.The material differed in one important respect from that used in a similar study by Dixon (1961). His samples were negative by direct plating. Those used in the current investigation were positive when directly plated and were, therefore, more heavily infected with salmonellas. Twenty years ago, major outbreaks of salmonellosis were more common and it was possible to record the duration of infection with some accuracy (George, Harvey & Thomson, 1953). Such episodes are not now encountered so frequently and convalescent faecal samples are much more difficult to obtain through environmental health officers. The only faecal material available contained salmonellas by direct plating. Despite this, 9 out of 25 positive samples produced only 1 or 2 salmonella colonies per plate at the 6 h subculture. If convalescent specimens had been used, early subculture would probably have resulted in even less positive samples than recorded here.

While Table 1 presents the cumulative increase in salmonella recovery obtained by quadruple plating of river Taff specimens after 6 h incubation in Rappaport's medium, Table 3 confirms the superiority of a 24 h subculture with a single plating. The later achieved almost four times the number of isolations obtained by a 6 h plating. There is little evidence that early subculture from enrichment is a reliable method of salmonella recovery.

Duplicate plating of environmental samples at 24 h (Table 1) is more promising. The 24 h subculture is more convenient than the 6 h for laboratory routine. The technique is simple and the 16 % gain (Table 1) compares favourably with the 2·6 % gain obtained in an earlier study by subculture from Rappaport's enrichment at 24 and 48 h (Harvey, Price & Xirouchaki, 1979). In contrast to selenite F and Muller–Kauffmann tetrathionate, 24 h appears to be near the optimum timing for subculture from Rappaport. In the present series, 6 out of 38 environmental samples would have wrongly been reported as negative had duplicate plating not been done.

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