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A method of secondary enrichment for salmonellas independent of selectively toxic chemicals

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INTRODUCTION

Man's environment is constantly contaminated with salmonellas and the technical problems of their isolation from heavily contaminated samples are of a different order from those confronting most hospital bacteriologists.

As a public health bacteriologist becomes more experienced, so his awareness increases of the danger of reporting false negative results. Bacteriology is, at best, an inexact science. Our attempts at selective culture of a particular micro-organism are still imperfect, even when well tried techniques are used.

Each attempt to improve the technique of salmonella isolation (Harvey & Thomson, 1953; Harvey, 1956; Harvey, 1957; Harvey & Price, 1962; Harvey & Price, 1964) has emphasized that a report 'failed to find salmonellas' was more truthful than the usual wording 'negative for salmonellas'. In an article on salmonella epidemiology, when the incidence of salmonella contamination of a material is given and conclusions are drawn from the results, it is well to remember that these same conclusions are only as valid as the figures on which they are based. It is in such investigations particularly that the bacteriologist requires to use the best available isolation techniques.

During the examination of crushed Indian bone for salmonellas (Harvey & Price, 1962), it was accidentally discovered that the passage of a mixed growth of salmonellas and other organisms through a modified Craigie tube (Harvey & Price, 1961), increased the ratio of salmonellas to other bacteria present. This was, therefore, a method of enrichment independent of selectively toxic chemicals. The phenomenon was not a new one. It recalled earlier attempts to isolate salmonellas from a mixed flora (Carnot & Garnier, 1902; Carnot & Weill Hallé, 1915; Friedburger, 1919; Friedburger & Putter, 1920; Pijper, 1952; Ino & Graber, 1955). The technique does not seem, however, to have been used extensively in routine practice, probably owing to the development of efficient enrichment media, such as tetrathionate and selenite broths.

The present paper is concerned with demonstrating that, occasionally, selective agars reported as negative for salmonellas by *experienced* observers are in actual fact positive. The method of demonstration employs the technique discussed above.

MATERIALS

The samples examined in this investigation were of two types; abattoir swabs and sewage polluted river water.

Abattoir swabs, in our laboratory, are incubated in selenite F broth at 43° C. and are subcultured to deoxycholate citrate agar, brilliant-green MacConkey agar (Harvey, 1956) and de Loureiro's (1942) modification of Wilson & Blair's medium. Incubation of highly contaminated samples in selenite F broth at 43° C. inhibits the growth of proteus species and is very useful for the isolation of salmonellas from sewage, bakery swabs and abattoir swabs (Harvey & Thomson, 1953; Harvey & Phillips, 1955; Harvey, 1956; Harvey, 1957; Harvey & Phillips, 1961). The three selective agars are incubated at 37° C and examined after 24–48 hr. incubation. It often happens that some but not all of these selective agars are positive, although inoculated from the same selenite F broth. We were interested in the *apparently* negative plates as suitable material for investigating the new method of enrichment. The abattoir samples chosen for investigation were, therefore, mainly those specimens which were positive on one but not on all three solid selective media. There were, however, certain abattoir samples which were *apparently negative on all three selective agars* but which we wished to examine in some detail for particular epidemiological reasons. The abattoir swabs in Table 1 therefore were not consecutive specimens and were chosen somewhat arbitrarily.

The river water specimens were approximately 100 ml. in volume and 10 × 10 ml. were examined at a time. They were diluted with 100 ml. double strength selenite F broth and five of the inoculated broths were incubated at 37° C. and 5 at 43° C. Previous work had shown that it was advisable to use both temperatures in the examination of sewage-polluted river water (unpublished). After incubation, the enrichment broths were subcultured only to de Loureiro's modification of Wilson & Blair's medium. The plates were incubated at 37° C. for 48 hr. before being read. Any apparently negative Wilson & Blair plates were then examined by secondary enrichment. In this series (Table 2) the samples were consecutive and unselected.

METHODS

Each apparently negative selective agar was rubbed over with an ordinary throat swab to remove the surface growth. This growth was suspended in 0·2 ml. of saline in a bijou bottle. A sterile Pasteur pipette with a bulb at the distal end of the capillary stem (Fig. 1) was then partially filled, by suction with a rubber teat, with 0·2 % nutrient agar. The upper surface of soft agar, at this stage, was allowed to come up to the level of the neck of the pipette barrel. The bacterial suspension was next sucked into the bulb at the lower end of the stem underneath the soft agar and in contact with it. A small air space, introduced by suction below the suspension, enabled the lower end of the stem to be sealed in a flame. The bulbous portion of the capillary was necessary to accommodate the 0·2ml. of dense bacterial suspension, otherwise the pipette and its manipulation was identical with that originally described by Harvey & Price (1961). The charged pipette was placed upright in a test tube with a plug of cotton-wool on the bottom and the whole

incubated at 37° C. for 24 hr. During incubation the growth at the lower end of the pipette spread up the stem and appeared on the surface of the agar. This surface growth was subcultured to deoxycholate citrate agar, brilliant-green MacConkey agar and de Loureiro's modification of Wilson & Blair's medium for the abattoir samples. In the series of river water specimens only brilliant-green MacConkey and de Loureiro's medium were used for plating. The selective agars were incubated at 37° C. for 24–48 hr. The brilliant-green plates were examined at 24 hr. and the deoxycholate citrate agar and de Loureiro plates at 48 hr. The 48 hr. incubation of *both* these plates was particularly important. Suspicious colonies were picked and examined in the usual manner.

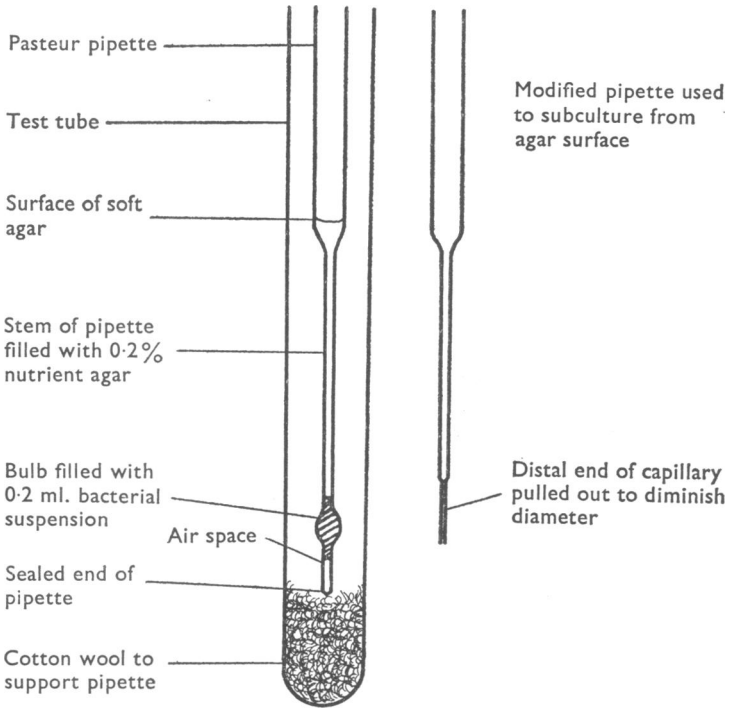

Fig. 1

RESULTS

The results are given in Tables 1, 2 and 3. It is obvious that apparently negative selective agars can often be converted to positive by this technique, and it is particularly noteworthy that six abattoir samples, subcultured from selenite broth in the preliminary examination on all *three* selective media, would have been reported as negative had the secondary enrichment technique not been used.

In order to show that the method was of general application, it was important to observe the range of serotypes which were successfully isolated. These serotypes are listed in Table 3. Both abattoir and river isolations are included. The method was found unsuitable for the isolation of *S. typhi*, *S. dublin* and *S. cholerae-suis*.

It was also unsuccessful in the isolation of the only strain of *S. paratyphi A* encountered in this laboratory during the investigation.

In a few samples the suspensions of growth from the selective agars were plated

Table 1. *Abattoir swabs*

Gross total of swabs positive by all techniques: 44.

Number of swabs which would have been reported as negative had the secondary enrichment technique not been used: 6.

Medium	No. of apparently negative plates examined	No. found positive after passage of growth suspension through pipette
Deoxycholate citrate agar	18	14
Brilliant-green MacConkey	27	19
De Loureiro medium	21	13

Table 2. *River water samples*

	ml.
Volume of sample	100
Total no. of 100 ml. samples examined	190
Total no. of samples positive without secondary enrichment	132
Total no. of samples positive by primary and secondary enrichment combined	158
'Negative' samples converted to positive by secondary enrichment	26

The combination of primary and secondary enrichment gives significantly better results than primary enrichment alone: $\chi^2 = 9{\cdot}8$; $P = 0{\cdot}002$.

Table 3. *Serotypes isolated from abattoir and river samples by pipette technique*

S. agama
S. anatum
S. blockley
S. bovis-morbificans
S. brandenburg
S. bredeney
S. butantan
**S. clifton* (salicin non-fermenting gelatin liquefying)
S. derby
S. eastbourne
S. give
S. heidelburg
S. kingabwa
S. meleagridis
S. oranienburg
S. panama
S. paratyphi B, phage types: 1, Dundee var. 1, 1 var. 1, Battersea
S. paratyphi B var. *odense*
S. poona
S. richmond
S. saint-paul
S. tennessee
S. thompson, phage type 15
S. typhi-murium, phage types: 12*a*, 4, 19, U 165, 1*a*, 32, 9, 12, untypable, 22

* The original description of *S. clifton* was of an organism fermenting salicin (Douglas & Taylor, 1954). The strain isolated in Cardiff failed to ferment 1 per cent salicin peptone water in 21 days.

before passage through the pipettes and these platings were compared with similar subcultures made after passage. The comparisons confirmed that enrichment was taking place.

DISCUSSION

It is unlikely that the 'negative' selective agars could be explained by any lack of efficiency of the plating media. Salmonellas are regularly isolated on all three media from a variety of materials. The brilliant-green MacConkey agar has acted as our standard plating medium for many years (Harvey, 1956) and the routine use of brilliant-green agars in Europe and America for salmonella isolation (Meat Hygiene, 1957) suggests that our own preference for this medium is not misplaced. The de Loureiro plates are regularly checked for their ability to grow a very wide range of salmonella serotypes (Harvey & Price, 1962) and have been found satisfactory for the isolation of *S. typhi* from stools, water supplies (Harvey & Price, 1964), sewage effluent and swabs from a clothes washing machine in a local mental hospital. Our deoxycholate citrate agar gives rich growths of salmonellas and shigellas from stools and when incubated for 48 hours gives almost as good results as brilliant-green MacConkey in the examination of abattoir swabs. It is an essential plating medium for the isolation of *S. dublin*.

Capillary pipettes, although essential to some techniques, for economy of sera (Harvey & Price, 1961; Harvey & Price, 1962), are fragile and are not best suited to routine purposes. It is interesting that a similar observation was made by Carnot & Garnier in 1902. We have, therefore, modified the above technique for the examination of animal feedingstuffs and have incorporated it into our laboratory routine. By means of this modification we have more than doubled our isolations of salmonellas from 25 g. quantities of animal feedingstuffs. The results of this latter investigation will be the subject of a further communication.

SUMMARY

A method of secondary enrichment is described suitable for the isolation of a wide range of salmonella serotypes from abattoir swabs and polluted river water. The technique does not employ selectively toxic chemicals.

REFERENCES

CARNOT, P. & GARNIER, M. (1902). Sur la technique des cultures en tubes de sable. *C.r. Séanc. Soc. Biol.* **54**, 748.

CARNOT, P. & WEILL-HALLÉ, B. (1915). Culture en 'tubes de sable' pour le diagnostic rapide de la fièvre typhoïde et le dépistage des porteurs de germes. *C.r. hebd. Séanc. Acad. Sci., Paris* **160**, 148.

DOUGLAS, S. HILDA & TAYLOR, JOAN (1954). Nine new salmonella species isolated from imported tortoises. *Mon. Bull. Minist. Hlth* **13**, 158.

FRIEDBERGER, E. (1919). Eine neue methode (Kapillarsteigmethode) zur Trennung von Typhus und Koli nebst allgemeinen Untersuchungen über das kapillare Steigvermögen der Bakterien im Filtrierpapier. *Münch. med. Wschr.* **66**, 1372.

FRIEDBERGER, E. & PUTTER, E. (1920). Weitere Versuche mit der Kapillarsteigmethode. *Münch. med. Wschr.* **67**, 398.

HARVEY, R. W. S. (1956). Choice of a selective medium for the routine isolation of members of the salmonella group. *Mon. Bull. Minist. Hlth* **15**, 118.

HARVEY, R. W. S. (1957). The epidemiological significance of sewage bacteriology. *Br. J. clin. Pract.* **11**, 751.

HARVEY, R. W. S. & PHILLIPS, W. P. (1955). Survival of *Salmonella paratyphi B* in sewers: its significance in investigation of paratyphoid outbreaks. *Lancet* ii, 137.

HARVEY, R. W. S. & PHILLIPS, W. P. (1961). An environmental survey of bakehouses and abattoirs for salmonellae. *J. Hyg., Camb.* **59**, 93.

HARVEY, R. W. S. & PRICE, T. H. (1961). An economical and rapid method for H antigen phase change in the salmonella group. *Mon. Bull. Minist. Hlth* **20**, 11.

HARVEY, R. W. S. & PRICE, T. H. (1962). Salmonella serotypes and arizona paracolons isolated from Indian crushed bone. *Mon. Bull. Minist. Hlth*, **21**, 54.

HARVEY, R. W. S. & PRICE, T. H. (1964). The isolation of *Salmonella typhi* from selenite enrichment media. *Mon. Bull. Minist. Hlth* **23**, 233.

HARVEY, R. W. S. & THOMSON, S. (1953). Optimum temperature of incubation for isolation of salmonellae. *Mon. Bull. Minist. Hlth* **12**, 149.

INO, J. & GRABER, C. D. (1955). Recovery of salmonella from contaminated cultures. *U.S. arm. Forces med. J.* **6**, 586.

LOUREIRO, J. A. DE (1942). A modification of Wilson & Blair's bismuth sulphite agar (stabilized stock solutions). *J. Hyg., Camb.* **42**, 224.

MEAT HYGIENE (1957). *Monograph Ser. W.H.O.* no. 33, p. 405.

PIJPER, A. (1952). Dimensional differentiation, filtration and separation of bacteria. *J. Path. Bact.* **64**, 529.