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The isolation of salmonellas from animal feedingstuffs

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In a previous publication (Harvey, Mahabir & Price, 1966) a motility technique was described for the isolation of salmonellas by secondary enrichment. The method was independent of selectively toxic chemicals and was applicable to the culture of a wide range of serotypes. The method was not original as it had been used by Gilbert & Fournier (quoted Carnot & Garnier, 1902) and by Carnot & Garnier (1902) as a primary enrichment process. By primary enrichment we mean culture of the original infected material in an enrichment medium (fluid or semi-solid). Secondary enrichment consists of further enrichment of an inoculum derived from the incubated primary enrichment broth. The use of the Craigie tube (1931) by Jones & Handley (1945) is an example of a differential motility technique used for secondary enrichment and the employment of a broth filled U-tube with arms divided by a sintered glass filter (Pijper 1952) illustrates the method used for primary enrichment. Recently a similar method has been described by Stuart & Pivnick (1965). In this case the motility technique was combined with the use of selectively toxic chemicals and the method used for primary enrichment.

The use of capillary pipettes filled with 0·15–0·2% nutrient agar in which agglutinating serum was incorporated had proved an invaluable method for isolating single salmonella serotypes from a mixture in an orderly and predetermined manner (Harvey & Price, 1962). In the later stages of this investigation (unpublished) we discovered that the migration of a mixture of salmonellas and other organisms through a column of 0·15% nutrient agar (without agglutinating serum) increased the relative proportion of salmonellas to other organisms appearing at the uninoculated surface of the agar. This observation aroused our interest and the technique became a profitable routine part of our examination of crushed bone for salmonellas. It was natural to extend the use of the method to the examination of abattoir swabs and polluted water samples and the results of this investigation were recently published (Harvey *et al.* 1966). The arrival in the laboratory of regular consignments of animal feedingstuffs infected with salmonellas suggested that the differential motility technique might be used for their examination and a trial of methods was, therefore, inaugurated.

MATERIALS AND METHODS

The materials examined were samples of commercial meat and bone meal, 250–300 g. in weight. All were produced by one firm. The firm's suppliers indicated that, as far as they were aware, the raw materials were of British origin.

Carnot & Garnier (1902) had found the apparatus used by Gilbert & Fournier

to be too complicated and too fragile for routine purposes. In our turn, we considered that the capillary pipettes, which had proved so useful in the Indian bone examination and in the secondary enrichment of salmonellas in abattoir samples and polluted water, to be lacking in strength for our routine practice. We, therefore, decided to modify the Craigie tube (Craigie, 1931), so that it could be used for our differential motility technique. A screw-capped 1 oz. (approximately 28 ml.) universal container was used. Inside this was placed a tube of dimensions 7×1 cm., open at both ends. Within this tube there was a glass rod 3·5 cm. in length with expanded ends, its distal end resting on the bottom inside surface of the universal container as in Fig. 1. The interior of the container was filled with 0·15 % nutrient agar to a depth of 5 cm. This concentration was chosen for convenience as it was obtained by diluting ordinary nutrient agar 1 in 10 with nutrient broth.

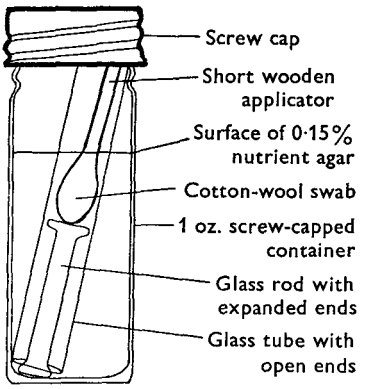

Fig. 1. Modified Craigie tube.

The technique used in this investigation was to culture four 25 g. portions of each separate specimen of feedingstuff. Each 25 g. sample was placed in an 8 oz. (approximately 220 ml.) wide-mouthed screw-capped jar and covered with 75 ml. of nutrient broth. The whole was incubated for 24 hr. at 37° C. After culture in broth, 75 ml. of double strength selenite F broth was added and the container and contents transferred to a water bath and incubated at 43° C. In this laboratory we have used this temperature for many years for the isolation of salmonellas from heavily contaminated samples such as sewage and abattoir swabs (Harvey & Thomson, 1953). This temperature is essential in our area for the examination of abattoir specimens, as without its use a statistically significant number of positive samples will be falsely reported as negative (unpublished observations). Subcultures were made from the selenite F broths at 24, 48 and 72 hr. on de Loureiro's (1942) modification of Wilson and Blair's medium. These plates were incubated for 48 hr. at 37° C. and suspicious colonies were picked and inoculated on the slope and in the condensation water of small agar slopes in bijou bottles for further investigation. These, after 6 hr. incubation at 37° C., showed sufficient turbidity in the water of condensation for satisfactory slide agglutination to be performed

with H agglutinating antiserum. Thus both the O antigen and one of the H antigens could frequently be identified 6 hr. after picking the colony. Biochemical reactions were seldom employed and complex screening media were rarely used. When the Wilson and Blair plates (inoculated from selenite broth after 24 hr. incubation) had been examined for salmonella colonies, they were subjected to secondary enrichment. This procedure was carried out on all such plates, whether negative or positive by the primary examination. The plates inoculated from selenite broth at 48 and 72 hr. were not subjected to secondary enrichment. The enrichment was carried out in the following manner: sterile cotton-wool swabs on wooden applicators 5 cm. long were rubbed over the surface of each plate to remove the surface growth. The charged swab was then introduced into the upper end of the 7 × 1 cm. tube inside the universal container (Fig. 1), with the cotton-wool resting on the upper end of the glass rod. The culture-coated swab was, therefore, covered with 0·15% nutrient agar. The inoculated modified Craigie tube was incubated at 37° C. for 24 hr. during which time growth appeared on the agar surface outside the 7 × 1 cm. tube. The growth was subcultured to brilliant green MacConkey agar (Harvey, 1956), and the plates were incubated at 37° C. for 24 hr. and examined for suspicious colonies in the usual way.

RESULTS

As this paper is intended to illustrate the use of a method of secondary enrichment for salmonella isolation in routine laboratory practice, it is best to consider the results obtained by primary and secondary enrichment separately.

Table 1. *Number of positive specimens obtained after subculture at various times*

	Subculture from selenite broth after incubation at 43° C. for		
	24 hr.	48 hr.	72 hr.
Positive plates	46	19* + 6†	18* + 5†
Total positive samples	46	52	57

* Plates from samples positive on previous subculture(s).
† Plates from samples not positive before.

Primary enrichment

It is evident (Table 1) that the majority of specimens are positive at the 24 hr. subculture from selenite F broth. Until the development of secondary enrichment, multiple subculture was regarded as being essential to efficient salmonella isolation. It has been used routinely in this laboratory since we first studied the survival of *S. paratyphi* B in sewers (Harvey & Phillips, 1955). In this technique it is not only necessary to consider the number of positives obtained at single subculture times, but also to observe the cumulative total positives found at 24 hr., 24 + 48 hr. and 24 + 48 + 72 hr. These results are also given in Table 1.

In this series, if the examination had ceased with the 24 hr. subculture, 11 positive samples would have been reported as negative.

Secondary enrichment

The results of secondary enrichment are given in Table 2.

In this series, four samples would have been reported as negative had the primary enrichment plates not been examined. All these four samples were negative at the 24 hr. subculture, but were positive either at 48 hr., or 72 hr., or at both times. All plates which were positive on the primary Wilson and Blair plates subcultured from selenite at 24 hr. were also positive when subjected to the secondary enrichment technique. If secondary enrichment alone had been relied on for the routine examination, only 3 % of the total possible positives would have been missed. These findings were important to the development of the routine examination that was ultimately adopted.

Table 2. *Secondary enrichment results*

Size of sample	25 g.
Total no. of samples	816
Total samples positive by primary enrichment alone	57 (42)
Total samples positive by secondary enrichment alone	133 (97)
Total samples positive by primary and secondary enrichment combined	137 (100)

Figures in parentheses show success rate as percentage of total positives obtained by primary and secondary enrichment combined.

Table 3. *Effect of sample size on the number of positives obtained*

Primary and secondary enrichment combined		Size of total sample
No. of positives obtained from 1 × 25 g. samples	30 (15)	25 g.
No. of positives obtained from 2 × 25 g. samples	43 (21)	50 g.
No. of positives obtained from 3 × 25 g. samples	54 (26)	75 g.
No. of positives obtained from 4 × 25 g. samples	62 (30)	100 g.
Total no. of 100 g. samples		204

Figures in parentheses show success rate as a percentage of the total number of 100 g. samples.

Effect of sample size on percentage success

It will be noted that in Tables 1 and 2, the sample size was 25 g. The effect of increasing the size of sample in stages up to 100 g. was also investigated. Four samples of 25 g. from each specimen of animal feed submitted by the manufacturer were separately examined. The first 25 g. was labelled A, the second B, the third C and the fourth D. The results are given in Table 3.

Table 3 shows that a fourfold increase in sample size doubled the percentage of positive results.

Serotypes isolated from animal feedingstuffs

As the infrequency of isolation of certain common serotypes from animal feedingstuffs is often quoted as evidence for their minor importance in the epidemiology of salmonellosis, it is worth while recording the serotypes encountered. In this connexion, it has been our experience that a detailed examination of a few specimens of the raw materials used as constituents for animal feed gives more useful information than the cursory examination of many samples. The cleavage of opinion as to the best approach to salmonella isolation techniques has been previously commented on by Felsenfeld (1945), though he did not commit himself as to preference. We record in Table 4 the information obtained separately by primary and secondary enrichment on the salmonella serotype content of the animal feedingstuff samples examined.

Table 4 records that *S. dublin* was not isolated and that *S. typhi-murium* was isolated three times. Eleven serotypes were isolated by secondary enrichment not found by primary enrichment. Two serotypes were found by primary enrichment only.

Table 4. *Salmonella serotypes isolated from* 100 *g. samples*

Serotype	No. of isolations by primary enrichment	No. of isolations by secondary enrichment
S. adelaide	0	1
S. alachua	1	0
S. anatum	0	3
S. binza	0	2
S. bonariensis	0	1
S. bredeney	0	2
S. california	5	6
S. cerro	1	1
S. cubana	0	3
S. derby	1	2
S. eimsbuettel	5	9
S. glostrup	1	1
S. havana	0	1
S. infantis	1	2
S. johannesburg	1	2
S. kentucky	0	1
S. kiambu	0	1
S. lexington	0	1
S. livingstone	0	2
S. meleagridis	1	2
S. mikawasima	1	2
S. minnesota	1	0
S. newington	3	3
S. oranienburg	0	2
S. senftenberg	7	7
S. singapore	0	2
S. tennessee	4	9
S. thomasville	1	2
S. typhi-murium	1	3
S. unidentified	0	1
No. of identified serotypes	16	27

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DISCUSSION

It will be noted that we have used preliminary culture of meat and bone meal samples in nutrient broth before culture in the fluid enrichment medium. This accords with the practice of Jepson (1957) and with our own experience of the isolation of salmonellas from dried material such as crushed bone. Not all salmonella serotypes are more easily recovered by incubation in broth before enrichment. *Salmonella typhi* in naturally polluted water is better isolated by immediate enrichment in selenite F broth. The isolation of *S. typhi* is, however, a separate problem.

The success of secondary enrichment is unlikely to be due to any inefficiency of the primary plating medium for the reasons given in our previous paper (Harvey *et al.* 1966). Whether Wilson and Blair, deoxycholate, S.S. agar or brilliant green MacConkey agar is used as primary plating medium, there is no doubt that the selective motility method of secondary enrichment greatly improves the results.

The best combination of primary plating medium (the selective agar used for subculture from selenite F broth) and secondary plating medium (the selective plating medium for subculture from the agar surface of the Craigie tube), has not yet been evaluated. The amount of labour involved in such complex trials is very great as large numbers of specimens and plates have to be examined. Wilson and Blair or brilliant green MacConkey are, however, eminently suitable for secondary plating media, and we have a strong preference for the latter as it is the only selective agar that adequately suppresses proteus species and it requires only 18–24 hr. incubation. It is true that positive results can be obtained with deoxycholate citrate agar used for secondary plating, but more labour is involved in picking colonies owing to the ease with which proteus species grow on this medium. Differentiation between salmonella and proteus colonies on deoxycholate plates can undoubtedly be improved by incubating them for 48 hr. The Wilson and Blair and brilliant green MacConkey agars plated from the Craigie tubes, in contrast to the deoxycholate plates, can be separated into negatives and presumptive positives on sight and suspicious colonies checked by slide agglutination. In some cases, the brilliant green MacConkey plates used for secondary plating from the Craigie tubes are almost pure cultures of salmonellas. Occasionally citrobacter strains on brilliant green MacConkey can be confused with salmonellas. This, however, rarely causes difficulty as these organisms can be readily distinguished from salmonellas by biochemical tests.

In the present investigation we observed the results obtained using Wilson and Blair as primary plating medium and brilliant green MacConkey as secondary plating medium. This combination was purposely chosen as, in our experience, differentiation of salmonella colonies is good on both these two media. Good differentiation is correlated with rapid diagnosis as no complex screening is necessary, apart from slide agglutination and ultimate confirmation of picked colonies by a salmonella reference laboratory. Rapid diagnosis is important as results are telephoned to the firm supplying the animal feedingstuffs as soon as positive O and

H slide agglutination is obtained with polyvalent sera. No errors have been made in the two years we have used the above technique.

The secondary enrichment technique was as quick as, and considerably more efficient than, the primary enrichment process. The method represents an attempt to employ a sensitive and selective technique (the two adjectives are not synonymous) in routine laboratory work. If a particular aspect of salmonella contamination of animal feedingstuffs required investigation such as the frequency of occurrence of *S. typhi-murium*, a more academic approach would be needed. For such research we think that the serological technique used in examining Indian crushed bone would be necessary (Harvey & Price 1962). By using a polyvalent H specific and non-specific serum from which the H phase I agglutinins of *S. typhi-murium* had been absorbed, the chances of recovery of the serotype might be improved and a truer estimate of its frequency obtained. A separate investigation is planned along these lines. In Indian material, the use of a progressive serological isolation process showed *S. typhi-murium* to be the sixth most common serotype encountered and the phage-types isolated often corresponded to those found in animals and man in the United Kingdom. We are not attempting to implicate Indian bones in the initiation of salmonellosis in this country. All inquiries indicate that manufacturers are afraid of using such material owing to the dangers of anthrax infection (Davies & Harvey, 1953, 1955; Jamieson & Green, 1955; Harvey, 1958). Examination of Table 4 raises some important questions. First, if the raw material is exclusively of British origin, why is *S. typhi-murium* not more evident? Why is the serotype distribution so exotic? Is there perhaps an admixture of imported material in the final product, or does it reflect plant contamination? The lack of isolation of *S. dublin* causes little surprise as, in our experience over a period of 10 years, *S. dublin* is poorly isolated on Wilson and Blair's medium. This applies both to our own laboratory prepared medium and to the main commercial brand of Wilson and Blair obtainable in this country. As we have not tested other formulae of Wilson and Blair we would hesitate to be sweeping in the interpretation of this phenomenon. Nevertheless, it should be taken into account in assessing the accuracy of figures showing the incidence of *S. dublin* infection.

Lastly, the dependence of successful isolation on the size of sample is well illustrated by Table 3. This is no new phenomenon, but was shown to be important in the isolation of salmonellas from American spray dried egg (Report, 1947), and more recently in the examination of animal feedingstuffs by Dutch workers (Jacobs, Guinée, Kampelmacher & van Keulen, 1963). The latter authors calculated that 10% of the bags of fishmeal imported into the Netherlands are probably contaminated with *S. typhi-murium*.

SUMMARY

A technique is described for the routine isolation of salmonellas from meat and bone meal samples used in animal feedingstuffs. The technique more than doubled the number of positives obtained by an orthodox method. Identical samples were used in the trial.

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