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Incubation at raised temperature of enrichment media, combined with secondary enrichment in Rappaport's medium, for the isolation of salmonellas from sewage

By D. TRICHOPOULOS, J. A. PAPADAKIS, D. KARALIS and P. VASSILIADIS

The Department of Hygiene and Epidemiology, University of Athens, Athens-609, *and the Hellenic Pasteur Institute, Athens*-602

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SUMMARY

A total of 50 duplicate Moore swabs were placed for 2 days, on five different dates, in 9–12 points of the Athens sewage disposal system.

Three methods of enrichment were used for the isolation of salmonellas. In the first method, one half of the duplicate swabs was incubated in Muller–Kauffmann's tetrathionate broth at 43° C. for one day. For the second method, a secondary enrichment was carried out in Rappaport's broth, made from the Muller–Kauffmann's broth and for the third method, the other half of the duplicate swab was cultured in Heart Infusion broth at 43° C. for 16–18 hr. after which a secondary enrichment was made in Rappaport's medium.

By use of these 3 enrichment procedures, 96% of the swabs were found to be positive for salmonellas. A total of 178 strains were isolated (an average of 3·7 strains per positive swab), belonging to 53 different serotypes (an average of 1·1 different serotypes per positive swab).

With the simple enrichment in Muller–Kauffmann's broth, only 72% of the swabs were found positive, and 68 strains of salmonellas belonging to 30 different serotypes were isolated. The secondary enrichment in Rappaport's medium made from the Muller–Kauffmann's broth produced 88% positive samples, and yielded 82 strains belonging to 34 different serotypes. Finally, with the secondary enrichment in Rappaport's broth made from the heart infusion broth, 92% of the swabs were positive and yielded 67 strains of salmonellas belonging to 27 different serotypes.

Although the last procedure yielded the greatest number of positive swabs, the method involving secondary enrichment in Rappaport's broth made from Muller–Kaufmann's broth led to the isolation of the greatest number of strains and different serotypes, while the other two procedures were approximately equal in this respect.

Of the 178 strains isolated, 110 were recovered only by the procedures involving secondary enrichment in Rappaport's broth. The most frequently isolated serotypes were *Salmonella senftenberg* (33 strains), *S. typhimurium* including var-*copenhagen* (18 strains), *S. poona* (11 strains), *S. montevideo* (10 strains), etc. The following

23 serotypes were isolated for the first time in Greece: *S. adelaide*, *S. alachua*, *S. allerton*, *S. binza*, *S. bobo*, *S. butantan*, *S. gnesta*, *S. goelzau*, *S. haelsingborg*, *S. havana*, *S. hofit*, *S. ibadan*, *S. indiana*, *S. irumu*, *S. jodhpur*, *S. nienstedten*, *S. panama*, *S. pomona*, *S. poona*, *S. reading*, *S. schwarzengrund*, *S. stockholm*, *S. tournai*. Moreover, a new serotype, *S. athinai* was described.

INTRODUCTION

In recent years, considerable work has been done in the techniques of enriching salmonellas from various materials. Following the work of Harvey & Thompson (1953), who reported a better yield of salmonellas when selenite F broth was incubated at 43° C., several workers have used elevated temperatures of incubation with selenite broth and with Muller–Kauffmann's tetrathionate broth, with satisfactory results in both cases. Care should be taken, however, with the composition of the tetrathionate broth used, since McCoy (1962) observed that Rolfe's tetrathionate B broth incubated at 43° C. was lethal to salmonellas. This unfavourable effect was attributed by Harvey, Price & Hall (1973) to the fact that the Muller–Kauffmann tetrathionate broth contains only 0·018 M tetrathionate, in comparison with 0·039 M tetrathionate used in Rolfe's B formula.

Another finding is, that in the case of material containing very few salmonellas and few other competing germs, pre-enrichment in broth results in a greater number of *Salmonella* isolations (North, 1961; Taylor, 1961; Vassiliadis *et al.* 1972).

Finally, several authors have reported that a secondary enrichment in selenite broth or tetrathionate broth also tends to increase the isolation of salmonellas from highly contaminated material. Similarly favourable results were also observed by Vassiliadis, Trichopoulos, Papadakis & Politi (1970), and Trichopoulos *et al.* (1972), when the secondary enrichment was made from selenite broth or Muller–Kauffmann's tetrathionate broth in Rappaport's medium.

The purpose of the present study was to determine the *Salmonella* serotypes prevalent in sewage from the Greater Athens agglomeration, and to evaluate the comparative efficiency for the isolation of salmonellas from sewage, of the simple enrichment in Muller-Kauffmann's tetrathionate broth incubated at 43° C. and of the secondary enrichment in Rappaport's broth from the former medium as well as from an enrichment in Heart Infusion broth.

MATERIALS AND METHODS

Materials

Between March and June 1973, a total of 50 duplicate Moore swabs were placed at 9–12 different points of the Greater Athens sewage disposal system. The points chosen for the placement of the swabs were in wide diameter sewers, conducting sewage from different parts of the city.

The swabs were made from several layers of strips of gauze, the approximate size of each swab being 15 × 45 cm. For each of the 50 samplings, two such swabs were tied together in the middle. Nine to 12 pairs of swabs were placed at the

selected sampling points, at intervals of 2–5 weeks. Each pair of swabs remained in the sewers for 2 days. On being removed from the sewer, each pair of swabs was placed in a separate, sterile, screw-capped jar. Two to 3 hr. from the time of their collection, the swabs arrived in the laboratory where the pairs were separated, each of the two strips being placed individually in a 300 ml. capacity, sterile, screw-capped jar.

Enrichment and selective media

The following enrichment media were used: Muller–Kauffmann's tetrathionate broth (MK broth); Difco Heart Infusion broth (HI broth) sterilized at 120° C.; and Rappaport's medium, slightly modified by Vassiliadis *et al.* (1970). Rappaport's broth was distributed in 5 ml. quantities in test tubes, autoclaved for 20 min. at 115° C., stored in the refrigerator and used within a month. For the preparation of the MK broth, the commercially available, dehydrated Muller–Kauffmann's improved tetrathionate broth base (Oxoid, CM343) was used. The iodine solution and 1/100,000 of brilliant green were added to the broth base in accordance with the formula given by Edel & Kampelmacher (1969), for a standardized method of *Salmonella* isolation.

The selective medium used was brilliant green-sulphadiazine-deoxycholate agar (BGSD agar). It was prepared with the commercially available, modified, dehydrated brilliant green agar (Oxoid, CM329), to which 2·5 g. of sodium deoxycholate per litre was added, and after boiling and cooling to 50°–60° C., 100 mg. of sodium sulphadiazine were introduced. The addition of sodium deoxycholate to the brilliant green was proposed by Papadakis *et al.* (1972), who found that this results in a marked inhibition of the growth of *Proteus* and of some coliforms, while allowing a luxuriant growth of salmonellas.

Cultural methods

To one of each of the pairs of jars containing the Moore swabs, 200 ml. of HI broth was added, while 200 ml. of MK broth was added to the jars containing the other halves of the swabs. Both of these broths were preheated to 45° C. The jars were then placed in an incubator at 43° C. A secondary enrichment was made after 16–18 hr. incubation, from the jars containing the HI broth, by transferring an inoculum into a tube of Rappaport's medium, with a 3 mm. loop (B/R). From the jars containing the MK broth, a subculture was made on a BGSD agar plate after 18–24 hr. (MK), as well as a secondary enrichment in a tube of Rappaport's broth, using a 3 mm. loop (MK/R).

The secondary enrichments in Rappaport's medium were incubated at 37° C. for 1 day, after which subcultures were made on BGSD agar. This selective medium was also incubated for one day at 37° C. From each positive plate, 4 colonies were inoculated in Kligler iron agar, and examined for their biochemical reactions and antigenic structure.

Table 1. Salmonella *isolations from sewage according to the enrichment procedure employed*

	Enrichment medium*		
	MK	MK/R	B/R
No. of positive swabs	36	44	46
No. of swabs examined	50	50	50
Positive swabs as percent of total	72	88	92

* MK = enrichment in Muller–Kauffmann's tetrathionate broth incubated at 43° C. for 1 day; MK/R = secondary enrichment in Rappaport's broth from MK enrichment incubated at 43° C. for 1 day; B/R = secondary enrichment in Rappaport's broth from enrichment in Heart Infusion broth at 43° C. for 16–18 hr. The growths from the enrichment and secondary enrichments were subcultured on brilliant green-sulphadiazine-deoxycholate agar.

RESULTS

Positive swabs in relation to the procedure employed

The number of swabs from which salmonellas were isolated is shown in Table 1. From this table, it can be seen that of the swabs enriched simply in MK broth at 43° C., only 36 out of 50 (72 %) were positive, whereas with the secondary enrichment in Rappaport's broth, made from MK broth and from HI broth, in 44 (88 %) and 46 (92 %) of the swabs respectively, salmonellas were isolated. It should be added that 48 of the 50 swabs examined (96 %), were positive for salmonellas with at least one of the procedures employed.

Isolation of serotypes and of strains of salmonellas, in relation to the procedure employed

As mentioned above, 4 colonies were examined from each of the positive plates of the selective medium. In many instances, more than one serotype was isolated from the samples enriched by each of the 3 procedures used. Moreover, different numbers of strains of the same serotype were isolated from different samples. Consequently, the number of serotypes, and particularly the number of strains isolated, was greater than the number of swabs examined (Table 2).

The distribution of the *Salmonella* strains, according to the techniques of enrichment by which they were isolated, is shown in Table 3. In Table 4, the efficiency of the three procedures used, with respect to the number of serotypes isolated per positive sample, is compared.

The serotypes and strains of Salmonella *isolated*

A total of 178 salmonella strains belonging to 53 different serotypes were isolated by the 3 enrichment techniques employed (see Table 5). Among the 53 serotypes found, 23 were isolated for the first time in Greece, and one was a new serotype belonging to sub-genus I, which has been named *S. athinai* (Vassiliadis, Trichopoulos, Papadakis & Le Minor, 1974).

The only strain of *S. haelsingborg* isolated, is a natural mutant which does not produce gas from glucose and has lost the nitrate, tetrathionate and thiosulphate

Table 2. Salmonella *isolations from sewage according to dates of sampling and the enrichment procedures used*

Date	No. of strains isolated by MK*	MK/R*	B/R*	All methods‡	No. of swabs examined
19. iii. 73	1 (1)†	10 (7)	14 (10)	22 (14)	9
2. iv. 73	5 (5)	12 (5)	8 (5)	17 (9)	9
16. iv. 73	17 (9)	20 (12)	11 (6)	42 (20)	10
21. v. 73	21 (15)	19 (10)	15 (11)	47 (23)	10
19. vi. 73	24 (17)	21 (14)	19 (21)	50 (28)	12
All days	68 (30)	82 (34)	67 (27)	178 (53)	50

* See footnote on Table 1.

† Figures in parentheses indicate numbers of serotypes isolated.

‡ Sometimes the same serotypes were isolated on different days. The numbers of strains isolated by different methods from the same sample are not additive, since the same strain was often isolated by more than one method.

Table 3. *Distribution of the* Salmonella *strains isolated by each of the three enrichment procedures employed*

Enrichment medium* MK	MK/R	B/R	No. of strains isolated
+	+	+	3
+	+	−	16
+	−	−	45
+	−	+	4
−	+	+	13
−	+	−	50
−	−	+	47
			Total 178

* See footnote on Table 1.

Table 4. *Distribution of serotypes and strains per positive sample according to the enrichment procedure employed*

	Enrichment medium* MK	MK/R	B/R	Total
No. of swabs examined	50	50	50	50
No. of swabs positive	36	44	46	48
No. of serotypes isolated	30	34	27	53
Average no. of different serotypes per positive swab	0·8	0·8	0·6	1·1
No. of different serotypes per positive swab				
1	17 (47)	17 (39)	28 (61)	—
2	10 (28)	17 (39)	15 (33)	—
3	5 (14)	9 (20)	3 (6)	—
4	4 (11)	1 (2)	0 (−)	—
No. of strains isolated	68	82	67	178
Average no. of strains per positive swab	1·9	1·8	1·5	3.7

* See footnote on Table 1. (Percentages in parentheses)

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Table 5. *List of* Salmonella *serotypes and strains isolated*

(Number of strains in parentheses)

S. senftenberg (33)
S. typhimurium (15)
* *S. poona* (11)
S. montevideo (10)
S. braenderup (8)
S. give (6)
S. tennessee (6)
S. westerstede (6)
S. anatum (5)
S. paratyphi B (5)
* *S. stockholm* (5)
S. infantis (4)
S. thompson (4)
S. blockley (3)
* *S. havana* (3)
* *S. reading* (3)
S. sofia (3)
S. typhimurium var. *copenhagen* (3)
S. bredeney (2)
S. derby (2)
* *S. goelzau* (2)
* *S. ibadan* (2)
S. livingstone (2)
S. muenster (2)
* *S. nienstedten* (2)
S. oranienburg (2)
* *S. panama* (2)
* *S. tournai* (2)
S. abony (1)
* *S. adelaide* (1)
S. agona (1)
* *S. alachua* (1)
* *S. allerton* (1)
† *S. athinai* (1)
* *S. binza* (1)
* *S. bobo* (1)
* *S. butantan* (1)
S. emek (1)
S. enteritidis (1)
* *S. gnesta* (1)
S. goeteborg (1)
* *S. haelsingborg* (1)
* *S. hofit* (1)
* *S. indiana* (1)
* *S. irumu* (1)
* *S. jodhpur* (1)
S. kentucky (1)
S. kottbus (1)
S. meleagridis (1)
S. muenchen (1)
* *S. pomona* (1)
S. saint-paul (1)
* *S. schwarzengrund* (1)

Total: 53 serotypes, 178 strains.

* Serotypes isolated for the first time in Greece.
† New serotype.

reductases. Similar mutants of *S. typhimurium* have been isolated from natural sources, or have been produced in anaerobic cultures by selection in the presence of chlorate (Le Minor, Piéchaud, Pichinoty & Coynault, 1969).

All the other strains isolated had the usual biochemical properties.

DISCUSSION

Fifty Moore swabs which had been placed in sewers in the Greater Athens area, were examined for the presence of salmonellas by three enrichment techniques: enrichment in Muller–Kauffmann's tetrathionate broth (MK broth) incubated at 43° C., and secondary enrichments in Rappaport's broth from the former medium, as well as from an enrichment in Heart Infusion broth (HI broth) incubated at 43° C. By these methods, salmonellas were isolated from 48 of the 50 swabs examined (96 %).

Furthermore, from the positive swabs, 178 strains of salmonellas belonging to 53 different serotypes were isolated. That is, an average of 3·7 strains and 1·1 different serotypes was isolated per positive swab (Table 4).

Several investigators have isolated salmonellas from sewage, using elevated temperatures of incubation of the enrichment medium (mainly selenite F broth). For example, Leclerc, Catsaras, Savage & Eymard (1970) examined 139 samples of sewage in Lille by such a method, and found 121 of them positive for salmonellas.

The number of strains which they isolated was 367, an average of 3 per sample, but they found only 45 different serotypes. On the other hand, examining 18 samples of sewage in Lebanon, Nabbut (1973) found all the samples to be positive. He isolated 16 serotypes, but only 18 strains.

Of the three procedures used in the present examination, the secondary enrichment in Rappaport's medium from the HI broth at 43° C. yielded the greatest proportion of positive swabs (92 %), followed by the secondary enrichment in Rappaport's broth made from the enrichment in MK broth at 43° C. (88 %), while with the simple enrichment in MK broth at 43° C., only 72 % of the samples were positive (Table 1).

The difference between the results of the two secondary enrichment methods is not statistically significant ($P > 0{\cdot}1$), while the difference between the results of those techniques and the results of the simple enrichment in MK broth, is statistically significant ($0{\cdot}05 > P > 0{\cdot}01$).

With regard to the number of salmonella strains and serotypes isolated by each of the three enrichment methods used, it was found that although the technique of secondary enrichment in Rappaport's broth made from HI broth resulted in the highest percentage of positive samples, the secondary enrichment in Rappaport's broth made from MK broth resulted in the isolation of more serotypes and more strains. In this respect, the other two techniques were almost equivalent to each other. Thus, by the B/R method, 67 strains belonging to 27 different serotypes were isolated, by the MK/R method 82 strains belonging to 34 different serotypes were distinguished, while by the simple enrichment in MK broth, 68 strains belonging to 30 different serotypes were found.

The number of strains which were isolated by only one of the three enrichment procedures used was large and very close (Table 3). That is, 45 strains were isolated only from the MK broth, 50 only from the secondary enrichment in Rappaport's broth made from MK broth, and 47 strains were isolated only from the secondary enrichment in Rappaport's broth made from HI broth. The difference in the efficiency of the three methods in isolating strains of salmonellas is not statistically significant ($P > 0{\cdot}1$).

These findings emphasize the importance of using more than one method of enrichment, and may explain the small number of strains isolated by Nabbut (1973), who used only one enrichment medium. The relatively small number of different serotypes isolated by Leclerc *et al.* (1970) can perhaps be attributed to the same reason.

Of the 178 strains found in this study, 110 were isolated only by the procedures involving a secondary enrichment in Rappaport's broth (Table 3), whereas only 45 of the 178 strains were isolated by the simple enrichment method alone and were not recovered with the secondary enrichments. Thus, our previous observations on the favourable results of the secondary enrichment in Rappaport's broth for the isolation of salmonellas in other materials (Vassiliadis *et al.* 1970; Trichopoulos *et al.* 1972, and other publications) are supported by the results of this study.

With regard to the number of strains of different serotypes isolated by each of

the three enrichment methods used (Table 4), the greatest number was recovered by the secondary enrichment in Rappaport's broth made from MK broth (82 strains of different serotypes) while the other two procedures yielded about equal numbers (68 and 67). It is evident, therefore, that the procedure involving a secondary enrichment in Rappaport's broth made from HI broth, which resulted in the highest percentage of positive swabs (92%), missed several serotypes. Thus, in only 6·5% of the samples which were positive with this procedure, were 3 serotypes isolated, whereas with the other two methods, close to 25% of the positive swabs yielded 3 or 4 serotypes. The weakness of the secondary enrichment in Rappaport's broth made from HI broth, in isolating a large number of the serotypes present in the sewage, may be attributed to the fact that the HI broth favours the emergence of 1 or 2 serotypes in heavy numerical predominance, and this may be further accentuated by the Rappaport's broth. Indeed, Harvey & Price (1967) have already reported the possibility of such behaviour of different serotypes in the enrichment media.

Of the 53 serotypes isolated, the following 23 were isolated for the first time in Greece: *S. adelaide*, *S. alachua*, *S. allerton*, *S. binza*, *S. bobo*, *S. butantan*, *S. gnesta*, *S. goelzau*, *S. haelsingborg*, *S. havana*, *S. hofit*, *S. ibadan*, *S. indiana*, *S. irumu*, *S. jodhpur*, *S. nienstedten*, *S. panama*, *S. pomona*, *S. poona*, *S. reading*, *S. schwarzengrund*, *S. stockholm*, and *S. tournai*. Moreover *S. athinai* is a newly described serotype (Vassiliadis *et al.* 1974).

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