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Salmonella isolation with Rappaport–Vassiliadis enrichment medium seeded with different sized inocula of pre-enrichment cultures of meat products and sewage polluted water

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SUMMARY

A total of 574 samples, of seven different types, were examined for the presence of salmonellas. All the specimens were pre-enriched in buffered peptone water and enriched in Rappaport–Vassiliadis medium (RV medium). In one trial 0·1 ml of pre-enrichment culture of 497 samples (79 chicken carcasses, 228 specimens of minced meat, 100 pork sausages, 19 samples of dried powdered chicken meat, 11 specimens of faeces of healthy pigs and 60 samples of sewage polluted natural sea water) was seeded to 10 ml as well as to 100 ml of RV medium. With the first inoculum (ratio 0·1 : 10), 111 samples were found to contain salmonellas, while with the second inoculum (ratio 0·1 : 100), only 102 positive samples were detected. This difference is marginally significant ($P < 0·05$). In another trial, 0·1 ml, 0·2 ml and 0·5 ml of pre-enrichment culture of 162 specimens (71 chicken carcasses, 40 samples of sewage polluted sea water and 51 samples of sewage polluted river water) were in turn introduced to 10 ml of RV medium. With the 0·1 ml inoculum 93 positive samples were detected, while with the 0·2 and 0·5 ml inocula 93 and 88 positive samples were found. The differences are not statistically significant. In these trials the growth of competing organisms was minimal with ratios of inocula 0·1 : 10 or 0·1 : 100.

INTRODUCTION

It was found that an inoculum of 0·1 ml of pre-enrichment culture of meat products in buffered peptone water, introduced to 10 ml of Rappaport–Vassiliadis medium (RV medium) incubated at 43 °C (inoculum ratio 1 : 100) was significantly more efficient in the isolation of salmonellas than an inoculum of the size of 0·02 ml (inoculum ratio 1 : 500) (Vassiliadis *et al.* 1978, 1984). It was also observed that inocula of pre-enrichment cultures of minced meat, of the size of 0·5 ml and 1·0 ml to 10 ml of RV medium (inoculum ratios 1 : 20 and 1 : 10) were significantly less efficient than an inoculum of 0·1 ml (Vassiliadis *et al.*, unpublished data, 1978).

Using the R25 modification (Vassiliadis *et al.* 1970, 1976) of Rappaport's enrichment broth, Harvey, Price & Xirouchaki (1979) and Harvey & Price (1980, 1981), observed that the more suitable size of pre-enrichment culture added to 10 ml of R25 broth was 0·005 ml. These authors also observed that an increase of

the size of the inoculum to 0·02 ml and 0·1 ml of pre-enrichment to 10 ml of R25 medium was very slightly (and not significantly) less productive than the 0·005 ml inoculum. Recently Harvey & Price (1983), have employed RV broth incubated, as recommended, at 43 °C. They reported that in the isolation of salmonella from sewage polluted natural water, the optimal size of inoculum of peptone water culture was 0·5–10 ml of RV medium. On the other hand, Tongpim *et al.* (1984), using samples of minced meat artificially contaminated with *S. typhimurium*, pre-enriched in buffered peptone water, observed that a volume of 0·1 ml of peptone water culture added to 100 ml of RV medium (ratio 1:1000) was significantly more productive than a volume of 0·1 ml of pre-enrichment culture seeded to 10 ml of RV medium (ratio 1:100).

These discrepancies prompted the undertaking of the present study in which different inoculum ratios of pre-enrichment culture in RV enrichment medium were employed, in the isolation of salmonellas from seven types of naturally contaminated products. The results observed are presented in the current paper.

MATERIALS AND METHODS

Samples. From September 1983 until January 1985, 574 samples were examined. The nature of the samples used is shown in Table 1. Among the chicken carcasses examined, 71 were imported frozen from four Western European countries, whereas the remaining 34 were refrigerated and came from five different owners of big poultry farms in Greece, each of them having his own poultry processing plant. Of the 228 samples of minced meat, 181 were of bovine meat imported frozen from Western European countries, while 47 were of minced pork meat, (31 samples imported frozen and 16 samples of local origin). The pork sausages and the faeces of healthy pigs were all of local origin. Thirty specimens of sewage polluted natural sea water were sampled from a sea bay (bay of Phaliron), 50–600 m from the mouth of the small river Kifissos which runs through the cities of Athens and Piraeus and is contaminated with human and other excreta from these towns. Another 30 samples of polluted sea water were taken from another nearby sea bay which receives the main sewer of Athens and Piraeus.

Fifty one samples of sewage polluted river water were also examined. These samples were taken from the river Pinios, 100–400 m beyond the outlet of the main sewer of the town of Larissa (a town of about 100000 residents, 340 km to the north of Athens). All the specimens were brought to the laboratory on Monday mornings (except for the frozen samples and the specimens of river water which were brought on Fridays and held in the refrigerator until Mondays).

Pre-enrichment. A pre-enrichment stage using buffered peptone water (Edel & Kampelmacher, 1973) was employed throughout this investigation. For this purpose: (*a*) 1 l of sea water was filtered through a 0·45 μm pore-size membrane. Each membrane was then introduced to a small jar containing 100 ml of buffered peptone water; (*b*) 250 ml of river water was added to 250 ml of double-strength buffered peptone water; (*c*) chicken carcasses were washed in sterile plastic bags with 500 ml buffered peptone water. The carcasses were removed and the wash was incubated, in big jars, at 37 °C for 18–22 h; (*d*) 50 g of minced meat or of finely cut pork sausages were added to 450 ml of buffered peptone water; (*e*) 25 g of dried

powdered chicken meat or 10 g of faeces of healthy pigs were added to 225 ml or 100 ml of buffered peptone water, respectively. The buffered peptone water was then incubated at 37 °C for 18–22 h.

Enrichment and selective media. The RV medium used in this study was prepared as described previously by Vassiliadis (1983) and Vassiliadis *et al.* (1976, 1981*a*, *b*). The selective plating medium was the modified brilliant green agar (Oxoid CM 329) (52g/l), in 950 ml of which, 2·5 g of sodium deoxycholate dissolved in 50 ml of distilled water were added before heating (Vassiliadis *et al.* 1979) (BGDA).

Inoculations and methods. All the inoculations were made to RV medium sterilized and held in the refrigerator, in glass screw-capped bottles of 500 ml capacity, for 18 days prior to inoculation. The RV medium was preheated to 43 °C before inoculation.

In a series of experiments, 0·1 ml of inoculum of the pre-enrichment culture was introduced to 10 ml of RV medium in test tubes, and 0·1 ml of the same inoculum to 100 ml of RV broth in small screw-capped glass jars (inoculum ratios of 1:100 and 1:1000, respectively). In another series of tests, 10 ml of RV medium received 0·1 ml, 0·2 ml and 0·5 ml of peptone water culture of the test material (inoculum ratios: 1:100, 1:50 and 1:20, respectively). All the tubes and jars containing the inoculated RV media were incubated at 43 °C for 48 h and subcultures were made, at 24 and 48 h to brilliant green deoxycholate agar (9 cm plates). The plates were incubated at 37 °C for 24 h and examined for the presence of salmonellas and for the degree of growth of competing organisms. Two suspicious colonies were picked to moist Kligler agar slopes for further identification. The growth of competing organisms on BGDA plates was graded subjectively, on a scale ranging from 0 to 4:0 indicates absence of competing flora or at most 50 colonies; 4 indicates growth over the whole plate; 1, 2, 3 means growth between these extremes. For each of the six types of specimens examined, the scores for competing organisms (0, 1, 2, 3 or 4) were averaged over the total number of samples (subcultures) of that type.

The statistical evaluation of the results was made using MacNemar's test for paired samples.

RESULTS

The total number, the nature of the samples examined and the number and percentage found contaminated with salmonellas, with at least one inoculum ratio, are recorded in Table 1.

The comparison of the efficiency of an inoculum of 0·1 ml from the pre-enrichment culture to 10 ml and to 100 ml of RV medium, respectively, is shown in Table 2. It should be added that 12 samples were positive with an inoculum of 0·1 ml of pre-enrichment culture to 10 ml of RV medium and negative with the same inoculum to 100 ml of RV medium; the reverse was true with only 3 samples. It should also be mentioned that with the 100 ml RV medium inoculated with 0·1 ml of peptone water cultures of chicken carcasses, 1 sample was positive after 24 h incubation of the RV medium and negative after 48 h, while 2 others were positive after 48 h and negative after 24 h incubation. Similarly from 3 samples of minced meat and from 1 sample of pork sausage, salmonellas were recovered only after 48 h incubation of the 100 ml RV medium. With the contaminated samples of sewage polluted natural sea water in the jars of 100 ml of RV medium, 2 samples

Table 1. *Salmonella isolations, after pre-enrichment followed by enrichment in* 18 *days old Rappaport–Vassiliadis medium seeded with different sized inocula*

(Subcultures on selective medium were made after incubation of the RV medium at 43 °C for 24 and 48 h.)

Samples	No. examined	No. positive	Positive (%)
Chicken carcasses	105	60	57·1
Minced meat	228	13	5·7
Pork sausages	100	7	7·0
Dried powdered chicken meat	19	13	68·4
Faeces of healthy pigs	11	0	0·0
Sewage polluted natural sea water	60	35	58·3
Sewage polluted river water	51	32	62·7
Total	574	160	27·9

Table 2. *Salmonella isolation with the use of Rappaport–Vassiliadis enrichment medium held in the refrigerator for* 18 *days and seeded with* 0·1 *ml of pre-enrichment medium in* 10 *ml and* 100 *ml, respectively*

		Inoculum/ratios	
Samples	No. examined	0·1 : 10 No. positive	0·1 : 100 No. positive
Chicken carcasses	79	48*	43*
Minced meat	228	13	12
Pork sausages	100	6	7
Dried powdered chicken meat	19	12	13
Faeces of healthy pigs	11	0	0
Sewage polluted natural sea water	60	32†	27
Total	497	111	102

* One chicken carcass was negative with the ratio 0·1 : 10 and positive with the ratio 0·1 : 100 while six other carcasses were positive with the ratio 0·1 : 10 and negative with the ratio 0·1 : 100

† Three more samples of sewage polluted sea water were positive for salmonellas with an inoculum of 0·5–10 ml of RV medium and negative with an inoculum of 0·1–10 ml of RV medium.

were found positive only after 24 h incubation, while 3 other samples were positive only after 48 h. In the tubes of 10 ml of RV medium which received 0·1 ml inoculum of pre-enrichment cultures of polluted sea water, 5 were found positive only after 24 h incubation and 6 only after 48 h incubation. With all the other specimens found to contain salmonellas, these organisms were recovered both after 24 and 48 h incubation of the RV media.

Table 3 shows salmonella isolations from each of three tubes containing 10 ml of RV medium inoculated with 0·1 ml, 0·2 ml and 0·5 ml of pre-enrichment culture, respectively.

The degree of growth of competing organisms in RV medium which received four different inoculum ratios of pre-enrichment culture, is presented in Table 4.

Finally, in Table 5, the serotypes and strains isolated during this investigation from six different types of samples, using four different inoculum ratios, are shown.

Table 3. *Salmonella isolation in* 10 *ml of* 18 *days old Rappaport–Vassiliadis medium inoculated with* 0·1 *ml,* 0·2 *ml and* 0·5 *ml, respectively, of pre-enrichment culture of three types of specimens*

Combination of findings according to inocula used			Types of samples			
0·1 ml	0·2 ml	0·5 ml	Chicken carcasses	Polluted sea water	Polluted river water	Total positive
+	+	+	42	13	28	83
+	+	−	2	4	2	8*
+	−	−	0	1	1	2*
−	+	+	1	1	0	2*
−	−	+	0	2	1	3*
−	−	−	26	19	19	64
Total positive			45	21	32	98
Examined (total: 162)			71†	40	51	—

* Among the positive samples, 5 were positive with an inoculum of 0·5–10 ml of RV medium and negative with an inoculum of 0·1 ml, while 10 others were positive with an inoculum of 0·1 ml and negative with an inoculum of 0·5 ml.

† 26 of these chicken carcasses were used only in this trial.

Table 4. *Degree of growth of competing organisms with different inoculum ratios*

Samples	Size of inoculum in Rappaport–Vassiliadis medium			
	0·1:10 ml	0·1:100 ml	0·2:10 ml	0·5:10 ml
Chicken carcasses	0·40* (105)†	0·71 (79)	0·66 (71)	2·23 (71)
Minced meat	0·58 (228)	0·63 (228)	1·70 (60)	2·53 (60)
Pork sausages	0·59 (100)	0·79 (100)	1·05 (10)	2·00 (10)
Faeces of healthy pigs	0·4 (11)	0·09 (11)	1·77 (11)	2·72 (11)
Sewage polluted natural sea water	0·82 (60)	0·64 (60)	1·55 (40)	2·21 (40)
Sewage polluted river water	0·51 (51)	not done	2·65 (51)	3·28 (51)

* The growth of the competing flora on brilliant green deoxycholate agar plates was graded on a scale ranging 0–4. Zero indicates absence of growth of competing organisms or growth of up to 50 colonies; 4 indicates growth on the whole plate; 1, 2 and 3 mean growths between these extremes. For each of the six types of specimens examined the scores of growth of competing organisms (0, 1, 2, 3 or 4) were averaged over the total number of samples of that type.

† Number of samples examined in parentheses.

DISCUSSION

It has been shown that an inoculum of 0·1 ml of pre-enrichment culture in buffered peptone water, seeded to 10 ml of Rappaport-Vassiliadis enrichment broth which is incubated at 43 °C (RV medium) (inoculum ratio 1:100) was significantly more efficient in detecting samples contaminated with salmonellae, than an inoculum of the size of 0·02 ml (inoculum ratio 1:500) (Vassiliadis *et al.*

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Table 5. *Serotypes and strains of salmonellas isolated from six different types of specimens with any of the different inocula*

Samples	Chicken carcasses	Minced meat	Pork sausages	Dried chicken meat	Polluted sea water	Polluted river water	No. of strains
Salmonella serotype							
agona	1	—	—	—	12	—	13
anatum	7	—	—	—	2	—	9
blockley	—	—	—	—	1	—	1
bovin morbificans	—	—	—	—	1	—	1
braenderup	—	1	—	—	1	—	2
chester	3	—	—	—	—	—	3
dublin	—	3	—	—	—	—	3
enteritidis	—	—	—	—	—	1	1
infantis	6	—	2	—	1	–	9
goldcoast	—	—	—	—	8	—	8
heidelberg	—	—	—	—	1	—	1
kottbus	—	—	—	—	1	—	1
lexington	—	1	—	—	–	—	1
livingstone	—	4	—	—	1	—	5
mbandaka	2	—	—	—	—	—	2
muenster	1	—	—	—	1	—	2
newport	—	—	—	—	1	—	1
ohio	—	—	—	7	—	—	7
oranienburg	—	—	—	—	3	—	3
orion	—	—	—	—	1	—	1
paratyphi B	—	—	—	—	5	2	7
*phaliron**	—	—	—	—	1	—	1
saint-paul	3	—	2	—	—	—	5
senftenberg	1	—	—	10	1	—	12
sofia	26	1	—	—	—	—	27
tennessee	—	—	—	—	2	—	2
thompson	1	—	—	—	—	—	1
typhimurium	7	3	3	—	7	13	33
t-*muriumvar.copenh.*	—	—	—	—	2	2	4
uphill	—	—	—	—	—	3	3
virchow	16	—	—	—	1	—	17
westerstede	—	—	1	—	1	15	17
3,10:1,6 (lack of phase I)	—	1	—	—	—	—	1
No. of strains	74	14	8	17	55	36	204
No. of serotypes†	12	7	4	2	22	6	

* *S. phaliron* is a new serotype.

† A total of 33 different serotypes were isolated from the six types of samples examined.

1978, 1984). It was also observed that a marked decline in the number of isolations of salmonellas from bovine minced meat was noted with a volume of 0·5 ml and 1 ml inoculum, of peptone water culture, to 10 ml of RV medium (Vassiliadis *et al.*, unpublished data). Using the R25 modification of Rappaport's medium, which is incubated at 37 °C, Harvey *et al.* (1979), and Harvey & Price (1980, 1981) found that the optimal inoculum of the pre-enrichment culture was 0·005–10 ml of R25 medium. Harvey & Price (1982) also examined the performance of strontium chloride B-malachite green enrichment medium, incubated at 43 °C,

with a range of different sized inocula. At this temperature of incubation of this medium, these authors observed a shift in optimal inoculum to 0·1 ml of pre-enrichment medium.

Recently Tongpim *et al.* (1984), reported that an inoculum of 0·1 ml of pre-enrichment culture to 100 ml of RV medium (inoculum ratio 1 : 1000) revealed significantly more positive samples, than a 0·1 ml sized inoculum of pre-enrichment culture to 10 ml of RV medium (inoculum ratio 1 : 100). The results in the current investigation, made on naturally contaminated samples, shows that an inoculum of 0·1 ml of pre-enrichment culture to 10 ml of RV medium revealed nine more samples positive for salmonellas (111 positive samples) than an inoculum of 0·1–100 ml of RV medium (102 positive samples). This difference is marginally significant ($\chi^2 = 4{\cdot}2$, $P < 0{\cdot}05$). This discrepancy with the findings of Tongpim *et al.* (1984) may be due to the fact that these authors used samples of minced meat artificially contaminated with one serotype.

It was indicated in the Results section that of the 111 samples from which salmonellas were recovered with enrichment in 10 ml of RV medium inoculated with 0·1 ml of peptone water culture, only 100 were found positive after both 24 and 48 h incubation, while 11 other samples were positive only after 24 h (5 samples), or only after 48 h incubation of the medium (6 samples). Although these irregularities were observed only when dealing with sewage polluted natural sea water, they show that it is safer to subculture the RV medium after both 24 and 48 h incubation at 43 °C. This discrepancy, however, was much more evident in the experiment in which 0·1 ml of pre-enrichment culture was added to 100 ml of RV medium.

Recently Harvey & Price (1983), compared the efficiencies of R25 (at 37 °C) and RV (at 43 °C) media, using as test material sewage polluted river water. They found that the optimal inoculum to 10 ml of RV medium was 0·5 ml, from the pre-enrichment culture of the natural water. For this reason, in the current investigation the efficiency of inocula of 0·1 ml, 0·2 ml and 0·5 ml of pre-enrichment cultures to 10 ml of RV medium were compared using as test material 162 samples of three different types. It was observed that the ratio 0·1 : 10 revealed slightly more positive samples than the ratio 0·5 : 10 (93 and 88, respectively) (Table 3). This difference is not statistically significant. The difference between these results and those of Harvey & Price may be due, in part, to the difference of 2 of the 3 test materials used in this study. In fact, Harvey & Price insisted that their findings concerning salmonella recovery from sewage polluted natural water must not be projected without further experiments to cover other material. However, in this investigation 51 samples of river water were also examined and no superiority of the 0·5 ml inoculum was detected. In fact, with this inoculum 29 samples of river water were found to contain salmonellas while with 0·1 ml inoculum, 31 samples were found positive (Table 3). An alternative explanation is that this discrepancy may be due to a difference in resistance towards the toxic effect of the enrichment medium of the strains isolated by Harvey & Price from sewage polluted natural water in Wales and the strains isolated from the test material used in the present study in Greece.

The inhibition of competing organisms in RV medium is similar in all trials in which 0·1 ml of pre-enrichment culture was introduced to 10 ml or to 100 ml of

RV medium. On the contrary, when the inoculum was 0·2 ml and especially, 0·5 ml of pre-enrichment medium to 10 ml of RV medium, the growth of competing organisms was much more evident (table 4).

Of the chicken carcasses examined, 57·1 %, were contaminated with salmonellas (Table 1). The carcasses of local origin were more heavily contaminated (97·1 %) than those imported (38·0 %). This difference may be due, in part, to the fact that the imported carcasses (500–900 g) were less than half the size of the chicken grown in Greece (1·600–1·900 g). Of the samples of minced meat examined, 5·7 % were contaminated. This percentage was higher in previous studies undertaken in Greece and especially in one investigation in which this type of meat was found contaminated in 22·2 % of the examined samples (Vassiliadis *et al.* 1978). This difference may be accounted for by the fact that frozen meat is now imported mostly from countries of the European Economic Community, while in the past, most of this frozen food was imported from South American countries. Of the samples of pork sausages, 7 % were found to contain salmonellas. In previous years, 26·7 % (Vassiliadis *et al.* 1981*c*), and even 46 % (Vassiliadis *et al.* 1981*d*) of the pork sausages examined were harbouring salmonellas. In this case, the most probable explanation is that, recently, a few big and modern piggeries were created in which the hygienic conditions are much better than those prevailing in the past. Finally, the high percentage (68·5 %) of contamination of the samples of dried powdered chicken meat is due to the fact, that for this product, only batches suspected of contamination with salmonellas were examined.

During this study a total of 33 serotypes and 204 strains of salmonellas were isolated. One of the serotypes, *Salmonella phaliron* (8:*z*:*e*,*n*,z_{15}), is a new serotype.

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