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Salmonella Testing of Pooled Pre-Enrichment Broth Cultures for Screening Multiple Food Samples

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Winton Hill Technical Center, The Procter & Gamble Company, Cincinnati, Ohio

Received for publication 10 December 1971

A method has been described for testing multiple food samples for *Salmonella* without loss in sensitivity. The method pools multiple pre-enrichment broth cultures into single enrichment broths. The subsequent stages of the *Salmonella* analysis are not altered. The method was found applicable to several dry food materials including nonfat dry milk, dried egg albumin, cocoa, cottonseed flour, wheat flour, and shredded coconut. As many as 25 pre-enrichment broth cultures were pooled without apparent loss in the sensitivity of *Salmonella* detection as compared to individual sample analysis. The procedure offers a simple, yet effective, way to increase sample capacity in the *Salmonella* testing of foods, particularly where a large proportion of samples ordinarily is negative. It also permits small portions of pre-enrichment broth cultures to be retained for subsequent individual analysis if positive tests are found. *Salmonella* testing of pooled pre-enrichment broths provides increased consumer protection for a given amount of analytical effort as compared to individual sample analysis.

Positive test results are relatively rare among the many samples of food ingredients and products which are analyzed for *Salmonella*. Therefore, a test procedure that gives a single negative answer for many negative samples offers an important saving in the analytical effort required for quality control. These studies support the conclusion that *Salmonella* pre-enrichment broths can be pooled for analysis without loss in sensitivity to *Salmonella* in the individual samples.

MATERIALS AND METHODS

Salmonella analyses were carried out as described in the *Bacteriological Analytical Manual* (BAM) (2) except for the pooling of pre-enrichment broths described in the following paragraph.

The procedure for *Salmonella* testing of pooled pre-enrichment broths is illustrated in Fig. 1. Samples are individually pre-enriched followed by transfer from multiple pre-enrichments to single selenite and tetrathionate broths at the enrichment stage. During pooling, a portion of each pre-enrichment is transferred to a sterile culture tube and retained at 4 C for later reference to the individual samples. If the *Salmonella* test of the pooled pre-enrichment broths is positive, individual tests on the retained samples can be made to determine which

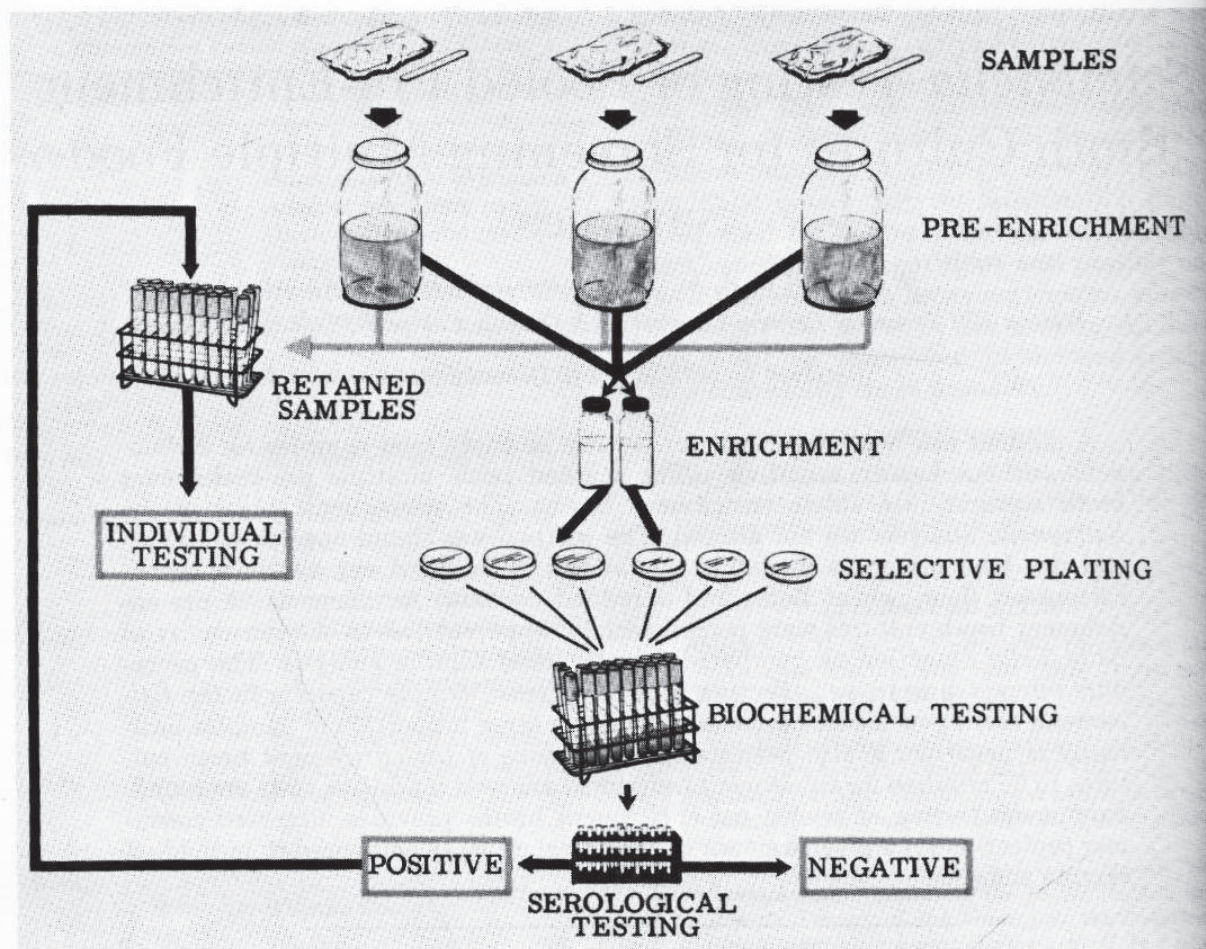
sample or samples contributed *Salmonella* to the pool.

Pre-enrichment broths consisted of 0.5% lactose broth for all foods except nonfat dry milk, for which sterile distilled water containing 0.002% Brilliant Green was employed.

Salmonella-positive test samples were prepared by blending the dry test food with one of three dry inocula. Inoculum 1 was a dry enzyme drain cleaner which had been found to contain multiple *Salmonella* serotypes including E₁,z₁₀; G,z₂₉ (*S. cubana*); and C₁,g complex. Inoculum 2 was a freeze-dried, skim milk suspension of serotypes *S. anatum*, *S. binza*, *S. tennessee*, *S. worthington*, *S. cubana*, *S. braenderup*, and a B₁,g complex. Inoculum 3 consisted of four laboratory isolates of serotypes G,z₂₉ (*S. cubana*); E₁,b; E₁,l complex; and C₁,l complex grown in a sterile aqueous suspension of cottonseed flour and freeze dried.

RESULTS AND DISCUSSION

Dried egg albumen, cocoa, and nonfat dry milk were inoculated with inoculum 1 at levels ranging from 6 salmonellae per 100 g to 3,000 salmonellae per g. Pre-enrichment broths of the *Salmonella*-inoculated samples were analyzed both individually and after pooling with 5, 9, and 14 pre-enrichments of uninoculated

FIG. 1. *Salmonella* testing of pooled pre-enrichment broths.TABLE 1. Effect of pooling pre-enrichment broth cultures on *Salmonella* recovery from food samples

Inoculation level ^a (per g)	Nonfat dry milk				Cocoa				Dried egg albumin			
	P ^b	P + 5N	P + 9N	P + 14N	P	P + 5N	P + 9N	P + 14N	P	P + 5N	P + 9N	P + 14N
Series I												
600	+	+		+	+	+		+	+	+		+
60	+	+		+	-	-		-	+	+		+
6	+	+		+	-	-		-	+	+		+
0.6	+	+		+	-	-		-	+	+		+
0.06	-	-		-	-	-		-	-	-		-
Series II												
3,000	+		+		+		+		+		+	
20	+		+		+		+		+		+	
0.2	+		+		+		+		+		+	

^a The most-probable-number estimate (1) of the original *Salmonella* inoculum (inoculum 1 containing serotypes E,z₁₀; G,z₂₉; and C,g complex) was 93,000/g with 95% confidence limits ranging from 15,000 to 380,000/g. The inoculation level represents a suitable dilution of inoculum 1, into an otherwise *Salmonella*-negative sample, to provide the levels as shown.

^b Abbreviations: P, positive sample inoculated with naturally contaminated dry enzyme drain cleaner; N, negative (uninoculated) sample; +, *Salmonella* recovery positive; -, *Salmonella* recovery negative.

samples. The positive inoculated samples were detected in pooled pre-enrichment broth cultures as effectively as when they were analyzed individually (Table 1). The level of inoculation

affected the recovery of *Salmonella* equally for both individual and pooled analyses. Detection of *Salmonella* in cocoa was less sensitive than in the other two test materials. A possibly sim-

ilar antimicrobial effect of cocoa on strains of *S. gallinarum* and *S. typhimurium* was reported by Busta and Speck (3).

Similarly, *Salmonella* was detected in wheat flour, coconut, and cottonseed flour samples without loss in sensitivity, with both inoculum 1 and inoculum 2, when single pre-enrichments were pooled with from 9 to 24 pre-enrichments of uninoculated samples.

Pooled samples versus pooled pre-enrichment broths. Sillicker (4) reported a loss in sensitivity when several samples were pooled in a large container of pre-enrichment broth as compared to individual sample analysis. This is not surprising since, to be detected, the desiccated and debilitated *Salmonella* cells in a dried food sample must grow out in pre-enrichment broth in competition with other bacteria in the sample. If many food samples are pooled at the pre-enrichment stage, the salmonellae face competition from the flora of all the samples in the pool. If, on the other hand, the samples are individually pre-enriched, the actively growing salmonellae might be expected to compete more favorably with organisms introduced from other samples when pooled at the enrichment stage. In addition, pooling pre-enrichment broths avoids the hazards and inconvenience of handling very large flasks of culture.

A study was made of *Salmonella* detection in pooled samples and pooled pre-enrichment cultures in the presence of added interfering organisms, i.e., non-*Salmonella* bacteria having colonies that resemble *Salmonella* on the selective agar media. The study was carried out with nonfat dry milk and egg albumin, using inoculum 3. About 500 cells each of four interfering organisms, *Pseudomonas* sp., *Proteus* sp., a lactose-positive *Citrobacter freundii*, and a lactose-negative Bethesda-Ballerup were added at the pre-enrichment stage.

Pooled samples were prepared by placing nine 25-g *Salmonella*-negative samples and one 25-g inoculated sample together in a single jar containing 2,250 ml of pre-enrichment broth. A pool of 10 samples was considered to be a practical maximum for convenient handling. Pre-enrichment pooling was carried out as previously shown in Fig. 1 except that 1 positive and 14 negatives were included in the pool.

Table 2 compares pooling of samples and of pre-enrichment broth cultures for nonfat dry milk and dried egg albumin at six levels of *Salmonella* inoculation. The results show that a higher number of positives was recovered by pooling pre-enrichments rather than by pool-

TABLE 2. *Effect of pooling stage on Salmonella detection*^a

Inoculation ^b level (per g)	Nonfat dry milk		Dried egg albumin	
	Pooled samples ^c (No. positive 3 repli- cates)	Pooled pre-enrichments ^d (No. positive 3 repli- cates)	Pooled samples ^c (No. positive 3 repli- cates)	Pooled pre-enrichments ^d (No. positive 3 repli- cates)
840	3	3	3	3
420	3	3	3	3
17	2	3	3	3
8	2	3	0	1
0.33	3	2	0	1
0.07	0	3	0	2
Total	13/18	17/18	9/18	13/18

^a Approximately 500 cells each of *Pseudomonas* sp., *Proteus* sp., *Citrobacter freundii*, and Bethesda-Ballerup were added to the pre-enrichment broth of each procedure.

^b The most-probable-number estimate of *Salmonella* in inoculum 3 (containing serotypes G₂,₂; E₁, b; E₁, L complex; and C₁, L complex) was 2,800/g with 95% confidence limits ranging from 1,000 to 15,000/g. The inoculation level represents a suitable dilution of inoculum 3 into an otherwise negative sample to provide the levels as shown.

^c Pooled samples = one positive and nine negative samples.

^d Pooled pre-enrichments = pre-enrichment broths from 1 positive sample and 14 negative samples.

ing samples. In addition, pre-enrichment pooling yielded positives at lower levels of inoculation. Similar results were found in tests with *Salmonella*-inoculated cocoa, shredded coconut, and wheat flour.

The pooling of broth cultures permits small portions of each individual culture to be retained for subsequent analysis if a positive test is detected following enrichment, thus maintaining the individuality of each test sample. On the other hand, the direct pooling of food samples into pre-enrichment results in the loss of individual sample identity.

Number of isolates examined. Two or more colonies are picked from each positive selective plate for biochemical and serological testing by the BAM *Salmonella* method (2) to increase the chances of detecting *Salmonella*.

By following this procedure, 180 isolates could be tested if 15 samples were tested individually, whereas only 12 isolates could be tested at the same rate of picking from a pool of 15 samples.

To test the importance of the reduced number of isolates, 238 isolates were picked at

the rate of one colony per positive selective plate from the pooled pre-enrichment culture tests of the three food materials: dry milk, egg albumin, and cocoa. Forty-four per cent of the isolates were *Salmonella*. In a similar evaluation of the tests on flour and coconut, 60% of 72 isolates picked at the rate of two colonies per plate were *Salmonella*. The high proportion of *Salmonella* indicated that pooled pre-enrichment provided an adequate number of isolates to detect the presence of *Salmonella*.

Whether pooled pre-enrichments are less sensitive than individual tests in the presence of interfering organisms probably is not known. For the present, we would recommend that, if numerous non-*Salmonella* colonies that resemble *Salmonella* on selective agar media are encountered in the pooled test, the retained pre-enrichment cultures should be tested individually. Alternatively, problems with interfering organisms may warrant the use of direct selective enrichments, two selective enrichments, or possibly even a reduction in the incubation time of the pre-enrichment cultures.

Effect of volume of pre-enrichment broth transferred. The BAM method (2) for *Salmonella* detection in egg products calls for the transfer of 1 ml of pre-enrichment broth culture to 10 ml of enrichment broth. In the multiple pooling of pre-enrichment broth cultures that has been described, 1-ml quantities of cultures were transferred, generally, to a volume of enrichment broth determined by the number of samples to be pooled to maintain a fixed pre-enrichment-to-enrichment ratio. Thus, 150 ml of enrichment broth was used to receive 15 1-ml poolings, 100 ml for 10 1-ml poolings, etc. However, for routine laboratory operations, it is more desirable to dispense enrichment broth at a constant volume. Since the number of samples to be pooled may vary considerably from time to time, it is important to know whether variations in enrichment ratio would significantly affect the detection of *Salmonella*.

Accordingly, a study was undertaken to test the effect of 1/10, 1/50, and 1/100 enrichment ratios on the recovery of *Salmonella* in inoculated food systems. The study included evaluation for both pooled samples and pooled pre-

TABLE 3. Effect of enrichment ratio on *Salmonella* detection in inoculated food materials^a

Method of detection	No. positive/18 tests		
	Enrichment ratio		
	1/10	1/50	1/100
Pooled pre-enrichments	11	13	15
Pooled samples	9	9	9

^a Chi-square tests and tests for linear trend between the number of positive tests and the enrichment ratio indicated no significant differences at the $P = 0.05$ level.

enrichment broth cultures. Each pooled pre-enrichment test was comprised of 15 25-g samples, whereas each pooled sample test consisted of 10 25-g samples. Six different tests were included for each ratio. Table 3 summarizes the results of this study.

No significant differences in detection were noted among the three ratios tested as measured by the chi-square test and the test for linear trend. The tentative conclusion drawn is that the enrichment ratio over the range investigated and for the food samples tested (nonfat dry milk, dry egg albumin, and cocoa) was not critical to *Salmonella* detection.

We have also examined the effect of 1-ml and 2-ml transfer volumes of the pre-enrichment cultures on *Salmonella* detection and found no significant differences in recoveries. This finding has prompted the standard use of a 1-ml transfer for routine *Salmonella* analyses.

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Exhibit 1050
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Salmonella Testing of Pooled Pre-Enrichment Broth Cultures for Screening Multiple Food Samples

W. R. PRICE, R. A. OLSEN, AND J. E. HUNTER

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sample or samples contributed *Salmonella* to the pool.

Pre-enrichment broths consisted of 0.5% lactose broth for all foods except nonfat dry milk, for which sterile distilled water containing 0.002% Brilliant Green was employed.

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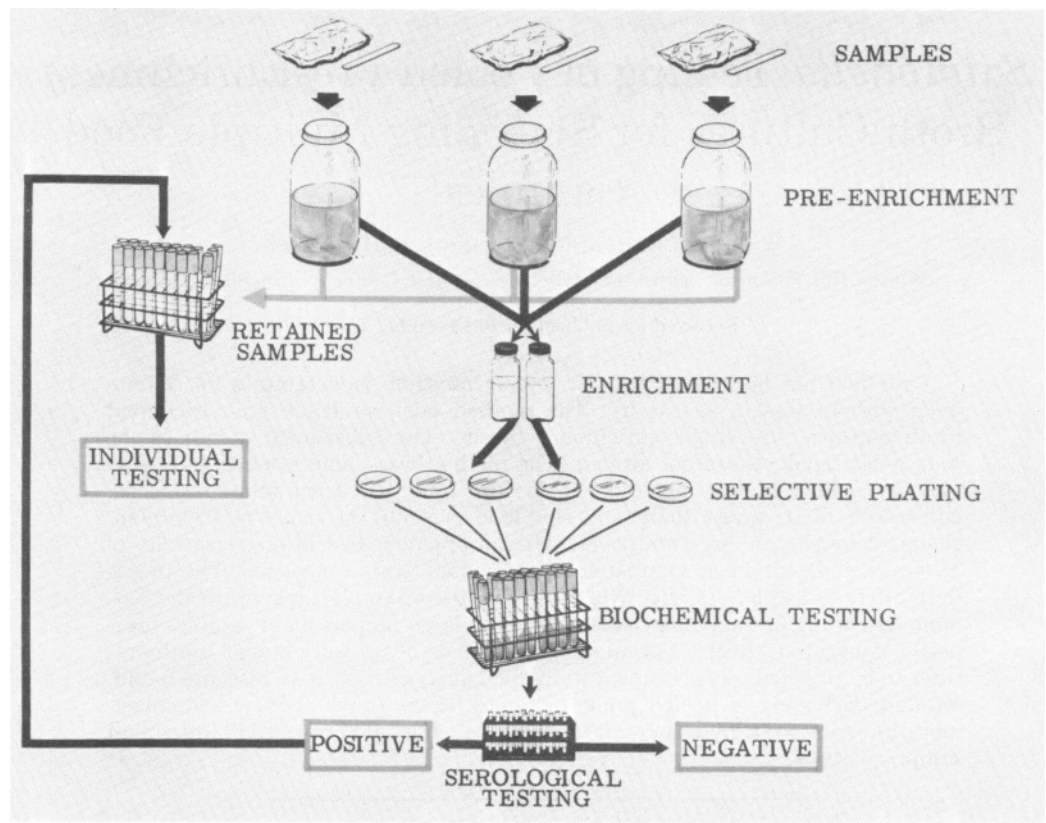


FIG. 1. *Salmonella* testing of pooled pre-enrichment broths.

TABLE 1. *Effect of pooling pre-enrichment broth cultures on Salmonella recovery from food samples*

Inoculation level ^a (per g)	Nonfat dry milk				Cocoa				Dried egg albumin			
	P ^b	P + 5N	P + 9N	P + 14N	P	P + 5N	P + 9N	P + 14N	P	P + 5N	P + 9N	P + 14N
Series I												
600	+	+		+	+	+		+	+	+		+
60	+	+		+	-	-		-	+	+		+
6	+	+		+	-	-		-	+	+		+
0.6	+	+		+	-	-		-	+	+		+
0.06	-	-		-	-	-		-	-	-		-
Series II												
3,000	+		+		+		+		+		+	
20	+		+		+		+		+		+	
0.2	+		+		+		+		+		+	

^a The most-probable-number estimate (1) of the original *Salmonella* inoculum (inoculum 1 containing serotypes E,z₁₀; G,z₂₈; and C,g complex) was 93,000/g with 95% confidence limits ranging from 15,000 to 380,000/g. The inoculation level represents a suitable dilution of inoculum 1, into an otherwise *Salmonella*-negative sample, to provide the levels as shown.

^b Abbreviations: P, positive sample inoculated with naturally contaminated dry enzyme drain cleaner; N, negative (uninoculated) sample; +, *Salmonella* recovery positive; -, *Salmonella* recovery negative.

samples. The positive inoculated samples were detected in pooled pre-enrichment broth cultures as effectively as when they were analyzed individually (Table 1). The level of inoculation affected the recovery of *Salmonella* equally for both individual and pooled analyses. Detection of *Salmonella* in cocoa was less sensitive than in the other two test materials. A possibly sim-

ilar antimicrobial effect of cocoa on strains of *S. gallinarum* and *S. typhimurium* was reported by Busta and Speck (3).

Similarly, *Salmonella* was detected in wheat flour, coconut, and cottonseed flour samples without loss in sensitivity, with both inoculum 1 and inoculum 2, when single pre-enrichments were pooled with from 9 to 24 pre-enrichments of uninoculated samples.

Pooled samples versus pooled pre-enrichment broths. Sillicker (4) reported a loss in sensitivity when several samples were pooled in a large container of pre-enrichment broth as compared to individual sample analysis. This is not surprising since, to be detected, the desiccated and debilitated *Salmonella* cells in a dried food sample must grow out in pre-enrichment broth in competition with other bacteria in the sample. If many food samples are pooled at the pre-enrichment stage, the salmonellae face competition from the flora of all the samples in the pool. If, on the other hand, the samples are individually pre-enriched, the actively growing salmonellae might be expected to compete more favorably with organisms introduced from other samples when pooled at the enrichment stage. In addition, pooling pre-enrichment broths avoids the hazards and inconvenience of handling very large flasks of culture.

A study was made of *Salmonella* detection in pooled samples and pooled pre-enrichment cultures in the presence of added interfering organisms, i.e., non-*Salmonella* bacteria having colonies that resemble *Salmonella* on the selective agar media. The study was carried out with nonfat dry milk and egg albumin, using inoculum 3. About 500 cells each of four interfering organisms, *Pseudomonas* sp., *Proteus* sp., a lactose-positive *Citrobacter freundii*, and a lactose-negative *Bethesda-Ballerup* were added at the pre-enrichment stage.

Pooled samples were prepared by placing nine 25-g *Salmonella*-negative samples and one 25-g inoculated sample together in a single jar containing 2,250 ml of pre-enrichment broth. A pool of 10 samples was considered to be a practical maximum for convenient handling. Pre-enrichment pooling was carried out as previously shown in Fig. 1 except that 1 positive and 14 negatives were included in the pool.

Table 2 compares pooling of samples and of pre-enrichment broth cultures for nonfat dry milk and dried egg albumin at six levels of *Salmonella* inoculation. The results show that a higher number of positives was recovered by pooling pre-enrichments rather than by pool-

TABLE 2. Effect of pooling stage on *Salmonella* detection^a

Inoculation ^b level (per g)	Nonfat dry milk		Dried egg albumin	
	Pooled samples ^c (No. positive 3 replicates)	Pooled pre-enrichments ^d (No. positive 3 replicates)	Pooled samples ^c (No. positive 3 replicates)	Pooled pre-enrichments ^d (No. positive 3 replicates)
840	3	3	3	3
420	3	3	3	3
17	2	3	3	3
8	2	3	0	1
0.33	3	2	0	1
0.07	0	3	0	2
Total	13/18	17/18	9/18	13/18

^a Approximately 500 cells each of *Pseudomonas* sp., *Proteus* sp., *Citrobacter freundii*, and *Bethesda-Ballerup* were added to the pre-enrichment broth of each procedure.

^b The most-probable-number estimate of *Salmonella* in inoculum 3 (containing serotypes G₂,₂₀; E₁, b; E₁, L complex; and C₁, L complex) was 2,800/g with 95% confidence limits ranging from 1,000 to 15,000/g. The inoculation level represents a suitable dilution of inoculum 3 into an otherwise negative sample to provide the levels as shown.

^c Pooled samples = one positive and nine negative samples.

^d Pooled pre-enrichments = pre-enrichment broths from 1 positive sample and 14 negative samples.

ing samples. In addition, pre-enrichment pooling yielded positives at lower levels of inoculation. Similar results were found in tests with *Salmonella*-inoculated cocoa, shredded coconut, and wheat flour.

The pooling of broth cultures permits small portions of each individual culture to be retained for subsequent analysis if a positive test is detected following enrichment, thus maintaining the individuality of each test sample. On the other hand, the direct pooling of food samples into pre-enrichment results in the loss of individual sample identity.

Number of isolates examined. Two or more colonies are picked from each positive selective plate for biochemical and serological testing by the BAM *Salmonella* method (2) to increase the chances of detecting *Salmonella*.

By following this procedure, 180 isolates could be tested if 15 samples were tested individually, whereas only 12 isolates could be tested at the same rate of picking from a pool of 15 samples.

To test the importance of the reduced number of isolates, 238 isolates were picked at

the rate of one colony per positive selective plate from the pooled pre-enrichment culture tests of the three food materials: dry milk, egg albumin, and cocoa. Forty-four per cent of the isolates were *Salmonella*. In a similar evaluation of the tests on flour and coconut, 60% of 72 isolates picked at the rate of two colonies per plate were *Salmonella*. The high proportion of *Salmonella* indicated that pooled pre-enrichment provided an adequate number of isolates to detect the presence of *Salmonella*.

Whether pooled pre-enrichments are less sensitive than individual tests in the presence of interfering organisms probably is not known. For the present, we would recommend that, if numerous non-*Salmonella* colonies that resemble *Salmonella* on selective agar media are encountered in the pooled test, the retained pre-enrichment cultures should be tested individually. Alternatively, problems with interfering organisms may warrant the use of direct selective enrichments, two selective enrichments, or possibly even a reduction in the incubation time of the pre-enrichment cultures.

Effect of volume of pre-enrichment broth transferred. The BAM method (2) for *Salmonella* detection in egg products calls for the transfer of 1 ml of pre-enrichment broth culture to 10 ml of enrichment broth. In the multiple pooling of pre-enrichment broth cultures that has been described, 1-ml quantities of cultures were transferred, generally, to a volume of enrichment broth determined by the number of samples to be pooled to maintain a fixed pre-enrichment-to-enrichment ratio. Thus, 150 ml of enrichment broth was used to receive 15 1-ml poolings, 100 ml for 10 1-ml poolings, etc. However, for routine laboratory operations, it is more desirable to dispense enrichment broth at a constant volume. Since the number of samples to be pooled may vary considerably from time to time, it is important to know whether variations in enrichment ratio would significantly affect the detection of *Salmonella*.

Accordingly, a study was undertaken to test the effect of 1/10, 1/50, and 1/100 enrichment ratios on the recovery of *Salmonella* in inoculated food systems. The study included evaluation for both pooled samples and pooled pre-

TABLE 3. Effect of enrichment ratio on *Salmonella* detection in inoculated food materials^a

Method of detection	No. positive/18 tests		
	Enrichment ratio		
	1/10	1/50	1/100
Pooled pre-enrichments	11	13	15
Pooled samples	9	9	9

^a Chi-square tests and tests for linear trend between the number of positive tests and the enrichment ratio indicated no significant differences at the $P = 0.05$ level.

enrichment broth cultures. Each pooled pre-enrichment test was comprised of 15 25-g samples, whereas each pooled sample test consisted of 10 25-g samples. Six different tests were included for each ratio. Table 3 summarizes the results of this study.

No significant differences in detection were noted among the three ratios tested as measured by the chi-square test and the test for linear trend. The tentative conclusion drawn is that the enrichment ratio over the range investigated and for the food samples tested (nonfat dry milk, dry egg albumin, and cocoa) was not critical to *Salmonella* detection.

We have also examined the effect of 1-ml and 2-ml transfer volumes of the pre-enrichment cultures on *Salmonella* detection and found no significant differences in recoveries. This finding has prompted the standard use of a 1-ml transfer for routine *Salmonella* analyses.

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