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and-Bone Meal of One 300-g Sample Versus
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Twenty-five meat-and-bone meal samples were analyzed for salmonellae, comparing a single 300-g to ten 30-g samples. Seventeen were positive using the larger sample; eighteen were positive with the smaller. The 300-g sample showed a significantly higher ($P < 0.01$) percentage of confirmed salmonellae at 2 days of incubation than at 1 day. The ten 30-g samples did not show changes at 2 days. At 2 days, the 30-g samples showed significantly fewer confirmed salmonellae than the 300-g sample; however, there was no difference at 1 day. Of 1,417 presumptive colonies picked, 1,215 (85.7%) were lysine decarboxylase-positive and 1,152 (81.3%) were agglutinated by one of the somatic antisera. There were no significant differences in diversity or total numbers of different somatic groups between the large and small samples.

The choice of sample size for microbial analyses depends on a number of factors such as size of available analytical equipment, size of original lot of material, particle size, and personal preference. It is axiomatic that no sampling scheme is perfect unless the whole lot is assayed destructively. Even then, success in finding the contaminating microorganisms depends on the efficiency of the assay procedures. Most sampling schemes are based on statistical techniques, taking into account the basic assumptions of homogeneity of contamination, random sampling methods, and a high degree of isolation efficiency.

A sample size of 25 g was recommended by the Food Protection Committee of the National Academy of Sciences (4) for routine work, with multiples of 25 g being used for foods with a high degree of consumer sensitivity. The Food and Drug Administration (7) also recommended 25-g samples. Thirty grams in 100 ml of enrichment medium was recommended as a sample size for isolating salmonellae from foods and feeds by Galton et al. (3). The United States Department of Agriculture (6) also recommended the use of a 30-g sample and 100 ml of enrichment. The American Public Health Association (1) suggested a 20-g sample and 80 ml of enrichment.

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The effect of compositing on the isolation efficiency for salmonellae from egg products was reported by Silliker (appendix C of reference 4). Some efficiency was lost when a composited sample was used; the suggestion was made that more work was needed on the effect of compositing on *Salmonella* recovery. The State/Federal Salmonella Program of the United States Department of Agriculture adopted the Salmonella Uniform Methods and Rules of the United States Animal Health Association (2). In this program, ten 30-g samples of meat-and-bone meal are collected from each rendering plant and returned to cooperating laboratories for *Salmonella* analyses. The samples are assayed individually. From the standpoint of labor, equipment, and time, it would be advantageous to pool the ten samples into one composite. Determining the effects of such compositing on *Salmonella* isolation efficiency was the purpose of the present study.

MATERIALS AND METHODS

Meat-and-bone meal samples. Twenty-five meat-and-bone meal samples were obtained from rendering plants in Pennsylvania, Ohio, Indiana, Wisconsin, and Iowa. One plant was sampled five times over a 2-year period and was known to harbor a considerable *Salmonella* population (represented by samples 13, 15, 20, 21, and 22). Sample 12 was also a known positive. The other 19 were selected without knowledge of previous history of *Salmonella* contamination. The samples were collected in ap-

proximately 2,000-g lots in plastic bags. Most of the samples were obtained from piles of meat-and-bone meal in bins or on the floor, but sometimes samples were taken directly from the conveyor lines. Each sample, prior to subdividing, was screened through a no. 12 standard sieve to remove the larger particles of teeth, bones, etc.

Subdividing technique. Approximately 650 g of the screened meat-and-bone meal was thoroughly mixed, and a 300-g sample was removed and placed into a 4,000-ml beaker. The remainder was used for ten 30-g samples in 8-oz (240 ml), wide-mouth, screw-cap jars and for ten 3-g samples which were placed in test tubes (approximately 1.9 by 15.2 cm).

Cultural procedures. A 3,000-ml amount of prewarmed (37 to 40 C) selenite-cystine broth was added to the 300-g sample. A 100-ml amount of the selenite medium was used for the 30-g and 10 ml for the 3-g sample. Samples were mixed thoroughly before incubation. The covers of the screw-cap bottles were placed on loosely. The tubes with the 3-g samples were covered with a 10-ml beaker. Incubation was at 37 ± 1 C for 24 ± 1 hr.

The incubated samples were mixed and streaked onto Brilliant Green agar plates. The 300-g sample was streaked onto four different plates, and each of the 30- and 3-g samples was streaked onto a single plate.

Preliminary confirmation. To perform a statistical evaluation, 40 presumptive colonies were picked from each sample. Five colonies were picked from each of the four Brilliant Green agar plates streaked from the enrichment medium of the 300-g sample and two colonies from each of 10 plates streaked from the 30-g enrichments. When 20 presumptive colonies were not available, the difference was made up by picking lactose-positive colonies. The lysine-iron slants were incubated for 24 ± 1 hr at 37 C. Production of a straw-colored butt was considered a negative *Salmonella* reaction, and these tubes were discarded. A neutral or alkaline butt, with or without H₂S production, was indicative of a presumptive positive *Salmonella* culture.

Final confirmation. The lysine-iron slants showing the presumptive *Salmonella* reactions were used for slide agglutination studies using grouped and individual somatic antisera. The antisera used were purchased from Difco and included the somatic groups A, B, C₁, C₂, D, E₁, E₂, E₃, E₄, F, G, H, I, poly A, poly A-1, poly B, poly C, poly D, and *Arizona* polydiphasic. The single factors 18, 21, 30, and 35 were also used. The reactions first were checked with the polyvalent antisera and whenever possible were further assigned to a somatic group.

RESULTS

Positive samples. The number of positive meat-and-bone meal samples is shown in Table 1. The 300-g sample gave 17 positives out of the total of 25, and the 30-g samples yielded 18 positives. The difference was not significant. Several samples were positive when one sample was used but not the other. Sample 4 was positive with the 300-g sample

TABLE 1. Efficacy of large and small samples for detecting positive samples of meat-and-bone meal

Meat-and-bone meal sample no. ^a	300-g Sample	Ten 30-g samples	Ten 3-g samples	No. of 30-g samples positive	No. of 3-g samples positive
1	+	+	+	10	4
2	+	+	+	8 (7) ^b	5
4	+	-	-	0	0
5	+	+	-	8 (9)	0
6	+	+	+	7	2
8	+	+	+	10	10
9	+	+	+	10	3
10	+	+	+	4	2
11	-	+	-	1	0
12	+	+	-	1	0
13	+	+	+	8 (7)	6 (5)
14	+	+	+	3 (6)	1
16	+	+	+	9 (10)	5
17	-	+	-	1	0
18	+	+	-	3	0
19	+	+	-	3	0
20	+	+	ND ^c	9 (10)	ND
21	+	+	ND	8 (9)	ND
22	+	+	ND	10	ND
Total positive	17	18	9		

^a Samples 3, 7, 15, 23, 24, and 25 were negative.

^b When the number of positive samples was not the same at 1 and 2 days of incubation, the 2-day results are given in parentheses.

^c Not done.

but negative with the ten 30-g samples, whereas samples 11 and 17 were positive with the 30- and negative with the 300-g sample. The remainder were either positive or negative with both sample sizes. If only the first two colonies picked from the first plates of the large samples were considered, 15 of the 17 samples were positive. An estimate of relative contamination is also shown in Table 1. Based on the numbers of positive samples obtained with the 3- and 30-g portions, sample 8 showed the highest level of contamination. Other samples with high levels of salmonellae were numbers 1, 2, 5, 6, 9, 13, and 16.

Positive colonies and somatic groups. Table 2 shows the numbers of lysine-positive cultures obtained from the different samples of meat-and-bone meal and the numbers of cultures which were confirmed as salmonellae by agglutination in somatic antisera. The results for the 300-g and ten 30-g samples can be compared directly because both had the same total weight of sample. The 3-g samples were not directly compared to the other samples. The table shows that there were more lysine-positive cultures isolated from the larger sample at both days. Of the total of 19 positive

TABLE 2. Number of positive cultures isolated from large and small samples of meat-and-bone meal

Meat-and-bone meal sample no.	Lysine-positive						Somatic group-positive					
	1 Day			2 Days			1 Day			2 Days		
	300-g	Ten 30-g	Ten 3-g	300-g	Ten 30-g	Ten 3-g	300-g	Ten 30-g	Ten 3-g	300-g	Ten 30-g	Ten 3-g
1	15	13	7	11	15	7	11	13	7	11	15	7
2	17	14	13	17	15	13	1	12	9	5	14	10
4	5	0	0	4	0	0	5	0	0	4	0	0
5	20	16	0	20	18	0	20	16	0	20	18	0
6	17	14	4	20	14	7	17	14	4	20	14	4
8	20	20	18	20	20	19	20	20	18	20	20	19
9	20	20	6	20	20	8	20	20	6	20	20	6
10	17	8	4	20	10	4	20	8	4	20	8	4
11	0	1	2	0	2	2	0	1	0	0	2	0
12	20	2	0	20	2	0	20	2	0	20	2	0
13	15	14	11	13	12	9	15	13	11	13	12	9
14	10	6	2	18	12	2	10	5	2	18	12	2
16	20	17	10	20	19	10	20	17	10	20	19	10
17	0	2	3	0	2	0	0	2	0	0	2	0
18	19	5	0	20	4	0	19	5	0	20	4	0
19	19	12	0	17	6	0	19	6	0	17	6	0
20	20	17	— ^a	20	20	—	20	17	—	20	20	—
21	17	13	—	20	14	—	17	13	—	20	14	—
22	20	20	15	14	20	15	19	19	15	14	20	15
Totals	291	214	95	294	225	96	273	203	86	282	222	86
Total colonies examined	317	246	139	300	266	149	291	214	95	294	225	96
% Confirmed	91.8	87.0	68.3	98.0	84.6	64.4	93.8	94.8	90.5	95.9	98.7	89.6

^a Not done.

samples at 1 day, the 300-g sample showed more lysine decarboxylase-positive cultures in 14, whereas only two of the ten 30-g samples had more positives. At 2 days, the larger sample had more lysine-positives in 12 samples compared to 4 for the smaller.

The larger sample also showed more somatic group-positive cultures than the smaller. Twelve samples at 1 day gave more positive cultures with the larger sample and four with the smaller. At 2 days of incubation, the larger sample was superior 11 times compared to 3 times for the smaller.

Chi square analyses of confirmation ratios. Table 3 shows the statistical analyses of the preliminary confirmation ratios (proportion of lysine decarboxylase-positive cultures to total presumptive Brilliant Green agar colonies). The 3-g samples in this case were compared to the 30-g samples, since proportions were used rather than absolute numbers per se. There was no significant difference between the single 300- and the ten 30-g samples at 1 day, but there was a highly significant difference ($P < 0.01$) at 2 days in favor of the larger sample. The 30-g sample also showed a significantly higher preliminary con-

TABLE 3. Chi square analysis of confirmation ratios of isolates from large and small samples incubated for 1 or 2 days

Time of incubation (days)	Sample size (g)	Lysine-positives/presumptive colonies	Somatic-positives/lysine-positives	Somatic-positives/presumptive colonies
		χ^2	χ^2	χ^2
1	300 vs 30	2.96	0.09	1.09
2	300 vs 30	31.5 ^a	5.38 ^b	11.8 ^a
1	30 vs 3	18.3 ^a	1.39	19.1 ^a
2	30 vs 3	21.0 ^a	12.0 ^a	31.7 ^a
1 vs 2	300	10.8 ^a	0.93	9.73 ^a
1 vs 2	30	0.02	3.99	0.03
1 vs 2	3	0.25	0.00	0.01

^a Significant difference $P < 0.01$ (critical $\chi^2 = 7.88$).

^b Significant difference $P < 0.05$ (critical $\chi^2 = 5.02$).

firmation ratio than the 3-g sample at both days. The 300-g sample showed a significantly higher confirmation ratio at 2 days than at 1. There were no differences in days of incubation for the 30- or 3-g samples.

The fully confirmed lysine ratios (proportion

of somatic-positive cultures to lysine decarboxylase-positives) are also shown in Table 3. There was a highly significant difference ($P < 0.01$) at 2 days of incubation in favor of the larger sample when the 30- and 3-g samples were compared. There was a less significant ($P < 0.05$) difference when 300-g sample was compared to the 30-g sample at 2 days (in favor of the 30-g) but not at 1 day. None of the other comparisons differed significantly.

Table 3 also shows the differences obtained in the overall confirmation ratios (proportion of somatic-positives to presumptive colonies). There were highly significant differences comparing the 300-g to the 30-g samples at 2 days (in favor of the 300-g sample), the 30- versus the 3-g sample at 1 and 2 days (in favor of the 30), and 1 versus 2 days with the 300-g sample (in favor of 2 days). The other differences were not significant. The fully confirmed overall ratios were nearly identical to the preliminary confirmed ratios.

Numbers of different somatic groups. The numbers of different somatic groups of the positive samples of the 300-g samples varied from none (samples 11 and 17) to seven for sample 9. The number of somatic groups in ten 30-g samples varied from none (sample 4) to six in samples 8 and 9. The total somatic groups were 39, 43, 48, and 45, respectively, for 1 day, 300-g sample; 2 days, 300-g sample; 1 day, 30-g samples; and 2 days, 30-g samples. None of these differences was statistically significant.

Somatic grouping of positive cultures. There were no significant differences in the isolation rates of any of the serogroups when 1 day of incubation was compared to 2 days, although a fairly high Chi square (4.21) was obtained with the H_2S -positive E_4 group, in favor of 1 day over 2 days with the 300-g sample. Comparison of the 300- versus the 30-g samples at 1 day of incubation showed a significant ($P < 0.05$) increase in the number of times that *Arizona* was recovered from the 30-g sample over the 300-g sample. *Arizona* was, however, recovered from only 2 of the 19 positive samples. At 2 days, there were significant increases in group B and E_2 isolations from the 30-g samples and in poly A-1 from the 300-g sample.

Comparison of large and small samples for isolation efficiency. A summary was made of the number of times that the large sample was equal to, worse than, or better than the smaller samples, using either one, two, three, or four of the Brilliant Green agar plates of the 300-g series for the comparison. When the criterion was the number of positive

samples out of the 19 samples tested, even with only one plate, picking five colonies, the 300-g sample was worse than the 30-g sample only three times and was the same 16 times. When all four plates were compared, the larger sample was better once, worse twice, and the same 16 times.

When the criterion of isolation efficiency was the number of diverse somatic groups obtained, the 300-g sample, with only one plate, was worse 10 times, the same 7 times, and better twice. This gradually improved until, with all four plates, the large sample was worse 7 times, better 5 times and equivalent 7 times. There did not appear to be any significant changes at the second day of incubation.

Lactose-positive salmonellae. No systematic studies were made on the number of times lactose-positive salmonellae could be isolated from the meat-and-bone meal samples. Some indication of their importance was obtained from the green colonies of the Brilliant Green agar plates which were picked to make up the total of 20 colonies. One group C_1 lactose-positive culture was obtained from the 30-g sample; three serogroups (*Arizona*, C_1 , and G) were obtained from sample 2; five C_1 cultures were obtained from sample 14. No further studies were made of these organisms.

DISCUSSION

It is conceivable that the size of sample could influence the recovery of one of a mixed group of microflora because of differences in growth rates due to inoculum size. These differences would be magnified when selective media were employed. Silliker's study (5) indicated that 16 of 389 lots of egg products were positive for salmonellae in both the 25-g individual sample and the 400-g composited samples. Twelve lots were positive in one or more of the 25-g samples and negative in the larger sample, whereas only one was positive in the large sample and negative in the small samples. The study reported here showed one lot positive with the 300-g sample and negative with the smaller samples; two of the 30-g samples were positive when the corresponding 300-g sample was negative. When the number of different somatic groups was compared at one day of incubation, the large sample was superior five times and the smaller samples were superior seven times. There was an indication that more somatic groups might be isolated when smaller samples were tested, but this could have been because a larger number of plates was examined in testing the small samples.

Statistical analysis of Silliker's data indicated that the agreement of the 25- and 40-g sample sizes would depend on the level of *Salmonella* contamination. With a low level of salmonellae, the two methods might disagree quite often, whereas with high levels they would generally agree. Similar results were found in this study.

The superiority of the larger sample over the smaller samples in the percentages of colonies which were lysine decarboxylase-positive (presumptive salmonellae) and somatic group-positive (confirmed salmonellae), as shown in Table 2, was perhaps an indication of the efficiency of a larger size sample in eliminating false-positive salmonellae. This might be of importance in some cases in which the number of samples assayed may be cumbersome; the weeding out of false-positives would save some time. However, there were indications that, at 1 day of incubation, the smaller samples recovered more types of salmonellae than the large samples.

In all the comparisons of the large and small samples, the same number of colonies was picked from each. To determine whether a sample was *Salmonella*-positive, only one plate (five colonies) from the large sample was needed to attain about the same efficiency as the smaller samples (total of 20 colonies). For determining the greater variety of serotypes, however, all four plates of the large sample were needed to approximate the efficiency of the ten smaller samples. The pooling of samples, as represented here by the large sample, would represent a considerable saving of time and labor by the analyst, even if all 4 plates and 20 colonies were analyzed.

The results indicated that somatic groups B, E₁, and *Arizona* occurred more frequently in the smaller samples and that group poly A-1 occurred more frequently in the larger samples. It should be noted that *Arizona* and the poly A-1 were found in only 2 of the 19 positive samples; E₂ was in 4, and B was in 7. Furthermore, when multiple Chi squares were done, there was a probability (when $P < 0.05$) that an average 1 out of 20 of the comparisons was significant; in reality, the high Chi square value may have been due to chance. Further studies of this nature would be needed to es-

tablish the validity of these values for Chi square. One way to obviate some of these difficulties is to set the α value lower, at 0.01. Under these conditions the chance of a random significant Chi square is only 1 in 100. Under these conditions, the only comparison among the above results that had a significant Chi square was group poly A-1; since this somatic group occurred in only 2 of the 19 positive samples, the validity even of this comparison may be questioned unless further studies on more samples are done.

The presence of apparently lactose-positive salmonellae in meat-and-bone meal deserves further study. The green or greenish-yellow colonies on the Brilliant Green plates are routinely disregarded; however, these studies indicate that they may need to be more carefully considered.

These data indicate that it is feasible to pool samples into one large sample. This will result in a considerable saving of labor and time with little loss of efficiency for determining the presence of salmonellae in a sample if two suspect colonies from the Brilliant Green agar plates are picked. To isolate the predominant serotypes from the meat-and-bone meal, more than 2 colonies should be picked, preferably 20 from 4 different plates.

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