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Research Note

Significance of Sample Weight and Enrichment Ratio on the Isolation of Naturally Occurring *Campylobacter* spp. in Commercial Retail Broiler Meat

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ABSTRACT

The goal of these experiments was to evaluate the efficacy of different meat to broth ratios for the isolation of *Campylobacter* spp. from retail broiler chicken meat. The evaluation included 25 g of meat enriched in 100 ml of Bolton broth (1:4 ratio, subsample A), 50 g in 200 ml (1:4, subsample B), 100 g in 300 ml (1:3, subsample C), and 150 g in 300 ml (1:2, subsample D). For 29 samples, another subsample (E) was evaluated at a 1:9 ratio. The results from 110 samples revealed no differences ($P > 0.05$) among subsamples (A through D) for the detection of *Campylobacter*-positive samples. By adding the results from subsamples A and B, the number of *Campylobacter*-positive samples was higher ($P < 0.05$) than that found based on results of subsamples A or B alone. However, the addition of the results from subsamples C and D increased the number of positive samples detected by only three. Subsamples C and D were the most contaminated, and contamination for subsamples A and B depended more on the original contamination of the meat than on the enrichment ratio. The mixing of the meat resulted in detection of more *Campylobacter*-positive samples than were found when the samples were not mixed before the subsamples were collected. No differences were found in the number of positive samples detected among subsamples A, B, C, or D based on product type. These results suggest that the linear extrapolation of positive results may not be appropriate for predicting the presence of *Campylobacter* spp. and that a 1:4 enrichment ratio with 25 g of meat is the most practical approach for the isolation of *Campylobacter* spp. from retail broiler meat.

Campylobacter jejuni and *Campylobacter coli* are important foodborne bacteria whose isolation from retail broiler chicken meat is achieved by enriching the sample before transfer to selective plating media. The standard sample tested is 25 g of broiler meat, which is enriched in nine times the volume of enrichment broth (1:9 ratio, wt/vol). Bolton broth is the most commonly used enrichment medium worldwide *(1, 2, 4, 5, 10, 16),* although the U.S. Department of Agriculture Food Safety and Inspection Service still recommends the use of Hunt enrichment broth *(18).*

The designation of a 1:9 ratio for enrichment purposes has never been adequately explained nor has the designation of 25 g as the optimal sample size. However, when using this sample size and ratio, the volume of enrichment needed to test several samples simultaneously becomes a serious limitation in food microbiology laboratories. Work with naturally contaminated retail broiler meat has revealed that a 1:4 enrichment ratio yields the same number of *Campylobacter*-positive samples as a 1:9 enrichment ratio. However, an increase in the number of positive samples, sometimes by more than 10%, can be achieved when duplicate samples are collected and analyzed *(11, 14).* This increase is not surprising because of the random nature of the distribution of bacterial pathogens in foods. For the isolation of *Listeria monocytogenes* from deli meat, enrichment ratios of 1:3, 1:5, and 1:10 have resulted in comparable growth rates *(21).*

For *Salmonella* in broiler meat, simulation studies have suggested that the prevalence and distribution of this pathogen increases in a nonlinear manner as a function of sample size, with 16% estimated prevalence in 25-g samples but 51% estimated prevalence in 100-g samples *(13).* However, no data are available on whether an increase in sample size would result in more *Campylobacter*-positive samples. Lower volumes of enrichment broth may be more economically feasible for laboratories with high throughput for the analysis of retail broiler meat. In addition, it is logical to assume larger amounts of meat per sample would result in detection of higher numbers of positive samples. We take for granted that 25 g is the standard amount of sample to be tested for isolation of *Campylobacter* spp. from retail broiler meat, but we do not know whether this is the most appropriate sample size for risk assessments.

The objective of this work was to evaluate whether an increase in sample weight would increase the number of naturally contaminated broiler meat samples detected as positive for *Campylobacter* spp. We also tested different

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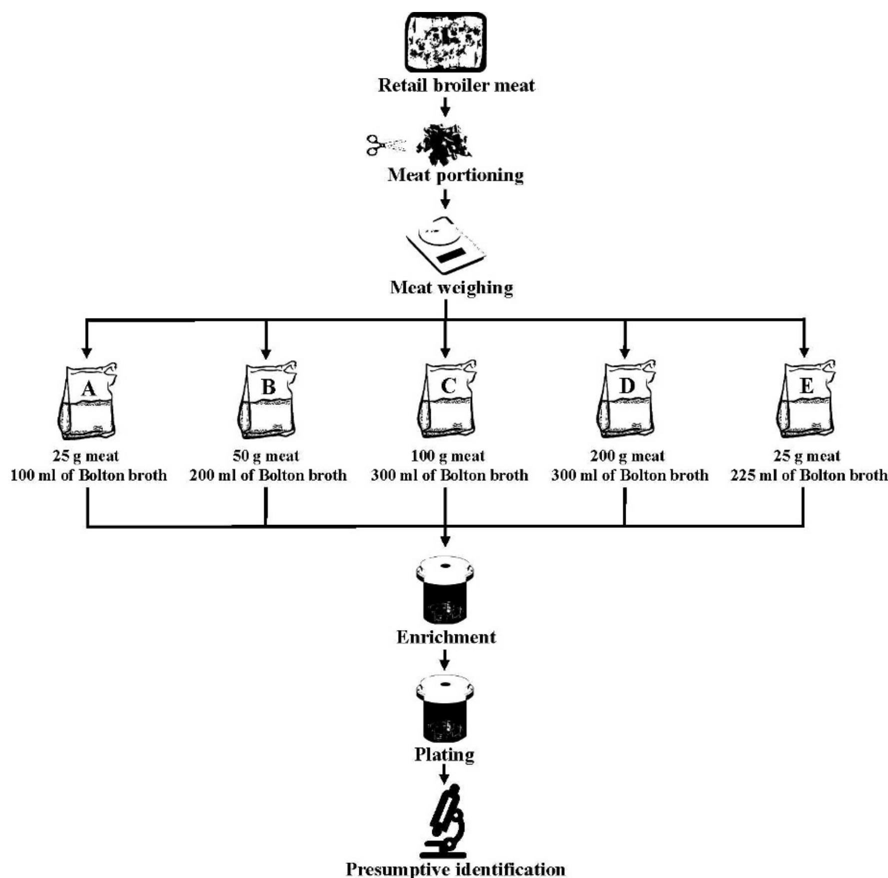

FIGURE 1. *Flow chart showing the methodology used to process, enrich, and presumptively identify* Campylobacter *spp. from retail broiler meat samples. For 57 samples, the pieces of meat from the same package (sample) were completely mixed before the subdivision of the meat into the different subsamples. No mixing was used for the rest of the samples (*n *= 53).*

enrichment ratios to allow for an increase in sample size without a major increase in the final volume of the enrichment sample. To better understand the impact of the mixing of the meat before testing, approximately half of the samples were tested without mixing the meat from different portions of the sample package. The results from different subsamples were analyzed both individually and pooled to determine whether more positive samples would be detected.

MATERIALS AND METHODS

Sampling protocol. Retail broiler meat samples ($n = 110$) were purchased in local food stores. These samples came from five major poultry companies and consisted of 70 skinless, boneless breasts and 40 skinless, boneless thighs. Each package of meat was considered a sample. Figure 1 shows the flowchart of the methodology used in these trials. For each sample, meat was cut in small pieces with sterile scissors, and for 57 samples the meat was thoroughly mixed with sterile forceps. From each sample, four subsamples (designated A through D) were divided as follows: subsample A was 25 g, subsample B was 50 g, subsample C was 100 g, and subsample D was 150 g. For 28 samples, another subsample (E, 25 g) also was incorporated in the evaluation. For the 57 samples for which the pieces of meat from different portions of the package were completely mixed before the subdivision of the meat into different subsamples, the mixing was done with sterile forceps and involved the intermingling of the pieces of meat to assure that each subsample incorporated segments from different parts of the package. For the rest of the samples ($n = 53$), no mixing of the meat was performed before the separation of the meat portions into subsamples.

Enrichment of the samples and *Campylobacter* isolation. After the meat for each subsample was weighed and placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI), the corresponding amount of Bolton broth (Acumedia, Lansing, MI) was added. Table 1 shows the amount of meat and the amount of Bolton broth used for each subsample. Bolton broth was prepared by the addition of the selective supplements (Oxoid Inc., New York, NY) and 5% (vol/vol) lysed horse blood. Samples were stomached for 1 min in a Stomacher 400 (Seward Laboratory Systems, Inc., Bohemia, NY) and enriched at 42°C for 48 h under microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2; Airgas, Radnor, PA) using an evacuation replacement system (MACSmics Jar Gassing System, Microbiology International, Frederick, MD) in anaerobic jars. After enrichment, samples (0.1 ml) were transferred onto modified Campy-Cefex (mCC) agar plates *(15)* with a 0.65-μm-pore-size cellulose membrane filter (Millipore Corp., Billerica, MA) *(20)*. The 0.1-ml drop of enrichment broth was deposited on top of the filter membrane, which had been placed on the surface of the mCC agar plate. The filter remained in contact with the surface of the agar plate for 15 min and then was removed with sterile forceps. Plates were incubated at 42°C under microaerobic conditions for 48 h and then observed for typical *Campylobacter* colonies (spiral morphology and darting motility) with phase contrast microscopy (Optiphot-2, Nikon, Tokyo, Japan). Isolates from a selected number of samples were individually stored at −80°C in tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) supplemented with 30% glycerol (vol/vol) and 5% blood.

Statistical analysis. Positive results from different subsamples were compared with a chi-square test as calculated with McNemar's test according to the following formula:

TABLE 1. *Description of the different subsamples used in these experiments, including amount of meat and enrichment broth*

Enrichment	Subsample:				
	A	B	C	D	E
Amount of meat (g)	25	50	100	150	25
Amount of Bolton broth (ml)	100	200	300	300	225
Ratio (meat:broth)	1:4	1:4	1:3	1:2	1:9

$$\chi^2 = \frac{(|b - c| - 1)^2}{(b + c)}$$

where b is a sample that has a positive test subsample (A through D) but a negative subsample E (considered the reference or control subsample) and c is a sample that has a negative test subsample (A through D) but a positive control subsample E. The accuracy and the kappa value (including observed agreement) were calculate from two-by-two tables and formulas as described by Hanrahan and Madupu *(8)*. The two-by-two tables were constructed using the ''tables'' command of SAS (version 9.1, SAS Institute, Cary, NC).

RESULTS

The analysis of five subsamples (A through E) from 28 samples revealed no differences in the number of *Campylobacter*-positive results among subsamples ($P > 0.05$). The comparison between subsamples A and E resulted in $\chi^2 = 1.0$ ($P = 0.31$). Nineteen samples were positive when subsamples A and E were added, and the concordance in the number of positive results between A and E (samples positive in both subsamples) was 79%. The evaluation of 110 samples revealed no differences ($P > 0.05$) in the number of *Campylobacter*-positive results among different subsamples (A through D). When the *Campylobacter*-positive results from subsamples A and E or A and B were added together, the number of *Campylobacter*-positive samples increased in comparison to the number of positive samples indicated by analysis of each subsample type alone ($P < 0.05$).

Of the total number of positive samples, 49% were positive based on results from all subsamples (A through D), five samples were positive based on only a positive subsample A, and one sample was positive based on only a positive subsample C. The accuracy and kappa values were highest for the comparison of positive results between subsamples A plus B (total positive) versus subsample A alone (Table 2). The highest percentage of *Campylobacter*-positive samples (80%) was obtained by the addition of A plus B plus C or A plus B plus C plus D (Table 3). However, no statistical increase in the total number of *Campylobacter*-positive samples was found by adding the *Campylobacter*-positive results from more than two subsamples (for instance, A plus B plus C).

The mixing of the meat resulted in numerically more *Campylobacter*-positive samples than were detected when the samples were not mixed; 8, 16, 19, and 21% more *Campylobacter*-positive samples were detected when subsamples A, B, C, and D, respectively, were collected after the original sample was mixed. Although there was an increase in the percentage of positive samples, there were no differences ($P > 0.05$) between mixed and nonmixed samples for subsamples A, B, and C. However, there were significant differences in the number of *Campylobacter*-positive samples detected between mixed and nonmixed samples for subsample D ($\chi^2 = 4.35$) (Fig. 2).

When analyzing the samples based on product type (breasts or thighs), there were no differences among positive samples for subsamples A, B, C, or D. For each product, the number of *Campylobacter*-positive samples detected was higher ($P < 0.05$) when adding the results from subsamples A plus B than for subsamples A or B alone (Table 3). The total number of positive samples (A plus B plus C plus D) by broiler company ranged from 57 to 100%.

DISCUSSION

The testing of foods samples is an important tool for controlling foodborne pathogens. Although several protocols have been designed for collecting samples from a food lot *(7)*, the actual amount of sample tested, 25 g, is standard for the testing of retail poultry meat in most countries worldwide *(1, 2, 4, 19)*. In some countries, this amount of product is routinely tested in two-class attribute acceptance plans, in which the number of positive samples below a

TABLE 2. Campylobacter-*positive results from 28 retail broiler meat samples tested with different meat:broth enrichment ratios*

Statistic	Subsamples[a]:					
	A	B	C	D	E	A+B[b]
No. (%) of positive samples	18 (64)	15 (54)	17 (61)	14 (50)	16 (57)	20 (71)
χ^{2c}	4.50	7.20	5.33	8.17	6.25	
Accuracy	0.93	0.82	0.89	0.79	0.86	
Kappa	0.84	0.63	0.76	0.57	0.70	
Agreement[d]	0.92	0.82	0.89	0.78	0.85	

[a] Subsamples: A, 25 g of meat enriched in 100 ml of broth; B, 50 g enriched in 200 ml; C, 100 g enriched in 300 ml; D, 150 g enriched in 300 ml; E, 25 g enriched in 225 ml.

[b] The addition of the positive results from subsamples A, B, C, D, and E yielded 20 (71%) positive samples, which were considered true positives.

[c] The chi-square value was calculated from the comparison between the results from subsamples A, B, C, D, or E with the results from subsamples A plus B.

[d] Observed agreement, independent from agreement by chance.

TABLE 3. *Results from the testing of 110 retail broiler meat samples for* Campylobacter *spp., grouped by product*

Product[a]	No. of samples	No. (%) of positive samples based on results from subsample[b]:					
		A	B	C	D	A+B	A+B+C
Breasts	70	47 (67)	39 (56)	44 (63)	39 (56)	53 (76)	54 (77)
Thighs	40	24 (60)	28 (70)	29 (73)	27 (68)	32 (80)	34 (85)
Total	110	71 (65)	67 (61)	73 (66)	66 (60)	85 (77)	88 (80)

[a] Boneless, skinless products. Comparison of subsamples A versus A + B: breasts, $\chi^2 = 6.0$ ($P < 0.01$), kappa = 0.79; thighs, $\chi^2 = 8.0$ ($P < 0.004$), kappa = 0.54; total, $\chi^2 = 14.0$ ($P = 0.0002$), kappa = 0.69.
[b] Subsamples: A, 25 g of meat enriched in 100 ml of broth; B, 50 g enriched in 200 ml; C, 100 g enriched in 300 ml; D, 150 g enriched in 300 ml; E, 25 g enriched in 225 ml.

stated value (*m*) are acceptable, and the number of positive samples with equal to or more than the stated value are unacceptable *(7, 9, 12)*.

In the United States, there is currently no microbiological standard for the presence or level of *Campylobacter* in retail poultry meat. The level of pathogen present in retail broiler meat is frequently low, around 0.7 CFU/g *(14)*. Therefore, a 48-h enrichment treatment is considered essential for reducing the number of false-negative samples *(11)*. In processed broiler meat, the number of *Campylobacter* cells present is already low, as demonstrated by the need to enrich the samples collected from processed broiler carcasses in the recent baseline studies by the U.S. Department of Agriculture *(3)*. If an industry standard were ever considered, it is reasonable to question whether the current standard amount of meat (25 g) and enrichment ratio for pathogen detection (1:9) are the most economical and the most sensitive approaches for the detection of *Campylobacter* spp. in retail poultry meat.

The first set of experiments included 28 samples in which several subsamples with different enrichment ratios were evaluated, with a control subsample at the 1:9 ratio. The subsamples included 25 g enriched in 100 ml of broth (1:4), 50 g enriched in 200 ml (1:4), 100 g enriched in 300 ml (1:3), and 150 g enriched in 300 ml (1:2). The results from this set of experiments helped us validate the 1:4 ratio as performing as well as the traditional 1:9 ratio *(14)*. In a similar study, the use of different ratios with a large amount of sample relative to the amount of enrichment broth did not alter the final results when testing for *L. monocytogenes* in deli meat *(21)*. The results from that set of experiments (with 30 samples) were similar to those from the present study, in which 110 samples were analyzed.

The McNemar test is used to compare the results from two related samples and is the recommended test for evaluating the results from microbiology analyses *(6, 17)*. A chi-square value of ≤ 3.84 indicates that the results from subsamples are statistically similar. Therefore, results equal to or below this cutoff value support the null hypothesis that the results from the subsamples compared are similar ($P > 0.05$). A limitation of comparing positive values from a subsample set with the total positive values from the sum of all subsamples is that the *b* cell in the two-by-two table is 0. Therefore, the false-positive rates for each subsample could not be calculated. Regardless of whether these comparisons yield significantly different values, the number of *Campylobacter*-positive samples increases by 5% to more than 10% when analyzing the results from the collection of two 25-g subsamples from retail poultry meat *(11, 14)*.

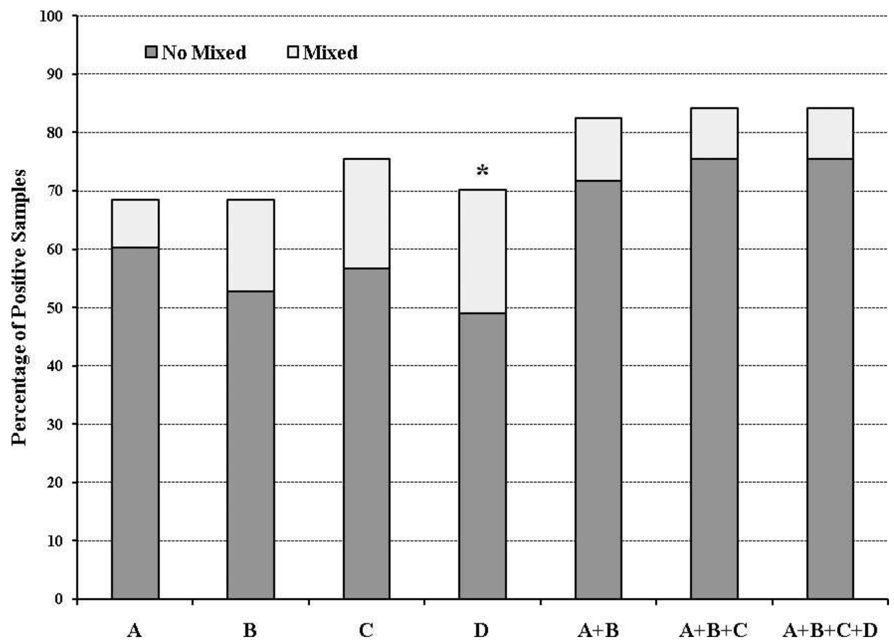

FIGURE 2. *Percentage of retail broiler meat samples positive for* Campylobacter *spp. The asterisk denotes significant differences between mixed and nonmixed samples for subsample D. Refer to Table 1 for details about the different subsamples.*

No scientific publications have included questions about whether sampling larger amounts of meat (more than 25 g) will result in detection of a higher number of positive samples. Based on the number of samples tested in these experiments, no increase in the number of positive samples detected should be expected by increasing the amount of meat to 50 g, i.e., twice the amount currently used for testing. Any enrichment ratio less than 1:4 will allow competing bacteria to hinder the isolation of *Campylobacter* spp. Consequently, 50 g of meat or more is not practically better nor will this larger amount yield better results than a combination of 25 g in 100 ml of enrichment broth.

The kappa value is a quantitative measure of the magnitude of agreement between observers. This value also could be interpreted as an inter-rater agreement for qualitative (categorical) observations *(8)*. The values for kappa range from +1 (perfect agreement) to −1 (complete disagreement), with 0 representing no agreement above that expected by chance. The kappa value for the comparison of the results from different subsamples with the number of positive samples by adding the results for subsamples A + B revealed that subsample A (25 g in 100 ml of broth, 1:4 ratio) is a good alternative to the traditional enrichment ratio of 1:9.

In conclusion, these results suggest that the linear extrapolation of positive results, a practice that has been used in microbial risk assessment for other foodborne pathogenic bacteria, may not be appropriate for predicting the presence of *Campylobacter* spp. in retail broiler meat. A 1:4 enrichment ratio with 25 g of meat is the best practical alternative for the isolation of *Campylobacter* spp. Because of the random distribution of *Campylobacter* cells and the low number of cells per gram of meat, the duplication of samples results in approximately 8 to 10% more positive samples detected, which may have an important impact when evaluating the safety of retail broiler meat.

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