Comparison of Methods for Recovery and Enumeration of *Campylobacter* from Freshly Processed Broilers

J. E. LINE,¹* N. J. STERN,¹ C. P. LATTUADA,² AND STEVEN T. BENSON²

¹U.S. Department of Agriculture, Agricultural Research Service, Russell Research Center, Athens, Georgia 30677; and
²U.S. Department of Agriculture, Food Safety Inspection Service, Athens, Georgia 30677, USA

ABSTRACT

Most traditional *Campylobacter* detection and enumeration procedures are difficult and time consuming. Estimations of *Campylobacter* populations by the most probable number (MPN) method are especially laborious. The objective of this collaborative study, performed in duplicate in Agricultural Research Service and Food Safety Inspection Service laboratories, was to compare two MPN procedures (utilizing different selective enrichment broths and plating media) to the direct plating technique for enumeration of *Campylobacter* from freshly processed (postchill, postdrip) broiler chicken carcasses. Results obtained from the direct plating of carcass rinse samples on Campy-cefex agar were not significantly different (P > 0.05) from an MPN procedure employing Hunt’s *Campylobacter* selective enrichment broth followed by recovery on modified *Campylobacter* charcoal differential agar. However, both of these procedures provided significantly (P < 0.05) better recovery than a second MPN procedure using Rosef’s selective enrichment broth followed by plating on Mueller-Hinton blood agar with antibiotics. The direct plating method offers a more simple, less expensive, more rapid alternative to traditional MPN procedures for estimating *Campylobacter* populations associated with freshly processed broiler carcasses.

Isolating campylobacters from food samples can be difficult. Enumerating campylobacters associated with raw processed chicken carcasses is especially troublesome because of the high levels of background microflora also found on the carcass. Numerous *Campylobacter* isolation methods have been reported (1, 4–6, 9, 12, 19). Qualitative isolation procedures usually involve recovery in a selective enrichment broth followed by plating the enrichment culture on selective agar. Quantitative techniques frequently rely on the most probable number (MPN) method for an estimation of *Campylobacter* numbers associated with a sample. Traditional *Campylobacter* MPN methods are both laborious and time consuming. Carcasses must be rinsed and the rinse suspension delivered into multiple replicate tubes (typically three or five) prepared at several serial dilutions. Incubation and antibiotic supplementation of the MPN tubes must be carried out under microaerobic conditions. After the proper incubation period, selective agar plates must be inoculated from each of the MPN tubes. These plates must then be incubated microaerobically and inspected for typical *Campylobacter* growth, which is then confirmed either biochemically or by latex agglutination. The number of positive tubes at each dilution is determined and the resulting estimate of *Campylobacter* CFU is computed from a statistical MPN table.

The direct plating method is a simpler enumeration procedure where portions of a carcass rinse sample are inoculated directly onto selective agar plates. The plates are incubated microaerobically and the number of typical *Campylobacter* CFU are counted on the plates. Typical colonies are confirmed by latex agglutination. The purpose of this study was to compare two MPN procedures (using different selective enrichment broths and plating media) to the direct plating technique for enumeration of *Campylobacter* from freshly processed (postchill, postdrip) broiler chicken carcasses. The Hunt-modified *Campylobacter* charcoal differential agar (MCCDA) MPN procedure employed Hunt’s *Campylobacter* selective enrichment broth (HEB) (10) followed by recovery on MCCDA (11). The second MPN procedure utilized Rosef’s selective enrichment broth (REB) (13) followed by plating on Mueller-Hinton blood agar with antibiotics (MHBA) (7). The direct plating procedure involved enumeration of *Campylobacter* recovered on Campy-cefex agar (18). Qualitative recovery in Bolton’s selective enrichment broth (Vidas Campylobacter product insert; bioMerieux Vitek, Inc., 595 Anglum Dr., Hazelwood, MO 63042) was also compared.

MATERIALS AND METHODS

Sample procurement and carcass rinse procedure. Fully processed broiler carcasses (postchill, postdrip) were obtained from three separate commercial processing plants in northern Georgia just prior to analysis. Samples were maintained on ice, transported to the labs, randomized, and analyzed within 2 h. A total of 24 carcasses were obtained and analyzed on four different sampling days within a 3-week time period. Each carcass was individually placed in a large sterile plastic bag (Cryovac, Duncan, S.C.). Buffered peptone water (250 ml) was added to each bag, and the carcasses were vigorously shaken for 2 min. Sample diluent was then recovered from each bag and was equally divided between the following two cooperating laboratories. All enumeration procedures were performed in duplicate by the U.S. Department of Agriculture Food Safety Inspection Service Special Projects and Outbreak Support Lab and the U.S. Department of Agriculture, Agricultural Research Service, Russell Research Center, Athens, Georgia 30677.
Agriculture Agricultural Research Service Poultry Microbiological Safety Research Unit both located in Russell Research Center in Athens, Georgia.

**FIGURE 1.** Schematic representation of MPN procedure utilized in this study using HEB.

Hunt-MCCDA MPN procedure. Portions of the carcass rinse (25 ml) were added to 25 ml of double-strength (2X) HEB. Four serial dilutions of three-tube MPNs (10 ml, 1 ml, 0.1 ml, and 0.01 ml) were prepared from the 1:1 mixture of sample in 2X HEB for quantitative recovery of campylobacters (Fig. 1). The remaining 46 ml of sample in HEB was added to a 1-quart resealable plastic bag (Reynolds, Richmond, Va.) and incubated and plated for qualitative recovery of campylobacters. The MPN tubes and the 46-ml sample bag were incubated at 37°C for 4 h. After 4 h the appropriate antibiotic supplement for HEB (cefoperazone; Sigma, St. Louis, Mo.) was aseptically added to all samples and controls. The samples were gassed again and placed at 42°C for 2 days. REB MPN tubes and the 46-ml sample bag were streaked on MHBA plates for isolation of campylobacters. The plates were incubated microaerobically at 42°C and examined at 48 h for growth of campylobacters.

**Direct plating procedure.** The direct procedure employs direct spread-plating of agar plates rather than MPNs for estimating campylobacter populations. Campy-cefex agar plates were prepared by the methods of Stern et al. (18) and held in the dark at room temperature for about 2 days prior to use. The original carcass rinse sample (0.1 ml) was surface plated in duplicate on the Campy-cefex agar. The plates were incubated as described earlier in resealable 1-gallon plastic bags flushed and filled with the microaerobic environment for 24 and 48 h at 42°C for quantitative estimation of Campylobacter populations associated with the carcass rinse samples. Additionally, a 1:1 dilution of sample (25 ml) in 2x Bolton enrichment broth (BEB; 25 ml) was prepared and incubated in a 1-quart plastic bag (with no modified atmosphere or agitation) at 37°C for 4 h. The bags were then placed at 42°C for 24 h and plated on duplicate Campycefex agar plates for qualitative recovery of Campylobacter spp.

**Campylobacter confirmation procedures.** Agar plates were examined after 24 and 48 h for presence of Campylobacter colonies. Representative colonies were subjected to a latex agglutination assay (Campylobacter culture confirmation test; Integrated Diagnostics, Inc., 1756 Sulphur Spring Road, Baltimore, MD 21227) to confirm the identity of the Campylobacter. Up to three typical colonies were tested as necessary to demonstrate the presence or absence of Campylobacter spp. Hazeleger et al. (8) found the latex agglutination assay capable of identifying about 83% of samples determined positive by traditional biochemical techniques.

**Statistical analysis.** MPN tubes from the Hunt-MCCDA and Rosef-MHBA methods found to contain Campylobacter were used to calculate MPNs using the appropriate tables. Plate counts were used to estimate Campylobacter populations from the direct plating procedure. Mean log Campylobacter populations per ml associated with the broiler carcass rinse samples were calculated and statistical analysis of the results (analysis of variance) was performed based upon a split-split plot design. Significant multiple comparisons were determined by the Student-Newman-Keuls test and correlations were determined using the Pearson product moment correlation procedure (Sigmastat; Jandel Scientific Software, San Rafael, CA 94912).

**Supplementary trials—direct plating agar comparisons.** In a subsequent study 20 carcasses were obtained as before and rinsed in 100 ml buffered peptone water. Each sample was split between the two cooperating laboratories. Campylobacter populations present in the rinse solutions were enumerated by the direct plating procedure. Portions (0.1 ml) of the carcass rinses were plated on duplicate plates of MCCDA, Campy-Cefex, and MHBA. Plates were incubated and counted as before. Additionally, a series of bags containing 25 ml of 2X HEB or 2X BEB were prepared and incubated with 25 ml of rinse. These bags were incubated as described earlier and plated on MCCDA, Campy-Cefex, and MHBA, and nonselective brucella agar for comparison of recovery of Campylobacter from the enrichment broths. An additional direct plating agar comparison was performed on 36 carcasses obtained at the beginning of a work shift from a Georgia processing plant.
These carcasses were analyzed by similar direct plating methods in the Poultry Microbiological Safety Research Unit lab only.

**RESULTS AND DISCUSSION**

Differences among the three methods did not depend on laboratories ($P = 0.42$); therefore, the results from 24 carcasses analyzed in the two laboratories were combined for a total of 48 observations. All of the carcasses (100%) were positive for *Campylobacter* by each of the three qualitative or quantitative methods evaluated; however, significantly fewer *Campylobacter* were recovered by the Roself-MHBA MPN procedure than either of the other two enumeration methods ($P = 0.001$). There was no significant difference in recovery ($P < 0.05$) between the Hunt-MCCDA MPN procedure and the direct plating procedure (Fig. 2). The direct plating procedure showed the carcasses to have a mean of about 80 *Campylobacter* CFU/ml of carcass rinse solution. A highly significant positive correlation coefficient ($P < 0.001$) of 0.889 was observed between the direct plating method and the Hunt-MCCDA MPN procedure (Fig. 3).

Several advantages of the direct plating procedure were observed in this study. A simple cost analysis of the three methods was performed. Materials cost for the tests (not including the latex agglutination assay) was calculated using labor estimated at about $20/h. The direct plating procedure was estimated to cost less than $6/sample, while the Hunt-MCCDA and Roself-MHBA MPN procedures cost about $31 and $35, respectively (Table 1). Fewer materials and less technician time were associated with the direct plating method because it was so much simpler to perform than handling 15 MPN tubes. The direct plating method can also give confirmed results 1 to 2 days sooner than the MPN methods. There was a significant positive correlation coefficient ($P = 0.01$) of 0.900 observed when direct plating results from the two laboratories were compared, indicating a high degree of reproducibility of the direct plating method.

Results from the 20 broiler chicken carcasses analyzed in the first direct plating agar comparison trial showed no significant difference in *Campylobacter* recovery between the two laboratories or between any of the three selective

**FIGURE 2.** Enumeration of *Campylobacter* (log CFU/ml) recovered from freshly processed broiler carcasses using MPN or direct plating methods.

**FIGURE 3.** Populations of *Campylobacter* isolated from freshly processed broiler carcasses by Hunt-MCCDA MPN or direct plating Campy-Cefex procedures (correlation determined by the Pearson product moment procedure).

**TABLE 1. Approximate cost and time comparisons of *Campylobacter* enumeration methods as performed in this study**

<table>
<thead>
<tr>
<th>Method</th>
<th>Media cost ($/sample)</th>
<th>Labor cost ($/sample)</th>
<th>Total cost ($/sample)</th>
<th>Technician time (min/sample)</th>
<th>Test duration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hunt-MCCDA</td>
<td>18.85</td>
<td>11.67</td>
<td>30.57</td>
<td>35</td>
<td>48–72</td>
</tr>
<tr>
<td>Roself-MHBA</td>
<td>23.27</td>
<td>11.67</td>
<td>34.94</td>
<td>35</td>
<td>72–96</td>
</tr>
<tr>
<td>Direct plating Cefex agar</td>
<td>3.03</td>
<td>2.67</td>
<td>5.70</td>
<td>8</td>
<td>24–48</td>
</tr>
</tbody>
</table>
agars. About 160 CFU/ml of carcass rinse solution were recovered from all of these carcasses; 100% were positive. Plates inoculated from the enrichment broth bags showed similar recovery of Campylobacter regardless of the agar type used. However, Campylobacter recovery from freshly processed chicken carcasses was enhanced by microaerobic growth in HEB as compared to aerobic growth conditions in BEB (Fig. 4).

The second agar comparison trial used 36 chicken carcasses obtained from the beginning of a work shift in a single processing plant. Less than 10 CFU/ml were recovered from these samples and 94% were positive by plating when results from all agars were combined (Table 2). Recovery on Campy-cfex, MCCDA, and MHBA revealed 67% (24 of 36), 64% (23 of 36), and 53% (19 of 36) of carcasses to be contaminated with Campylobacter, respectively. It was interesting to note that a differential count of Campylobacter and non-Campylobacter colonies on the selective agars showed Campy-cfex to be the least selective and MHBA to be the most selective of the three agars tested (Fig. 5). No contaminating bacterial colonies were observed on the MHBA plates.

Although it is possible that the direct plating technique might recover fewer sublethally injured microorganisms than the MPN procedure we found direct plating to be as sensitive as the MPN procedures for freshly processed carcasses with relatively high Campylobacter populations. Other researchers have found direct plating procedures to be superior to MPN techniques for enumerating campylobacters. Beuchat (3) determined direct plating on any of three selective agars to be better for recovering C. jejuni from frozen chicken meat than an MPN technique. Similar observations were made in a study enumerating C. jejuni from chicken meat stored at 5°C (2). Stern and coworkers (17), likewise, found direct plating onto Campy-BAP medium resulted in equivalent or greater recovery of C. jejuni from chicken and ground beef than did two different MPN procedures. Perhaps a greater difference would be observed between the two techniques when analyzing stressed organisms such as those Campylobacter found on retail broiler carcasses after several days storage at refrigeration temperatures. In a study by Stern (15) Campylobacter populations recoverable by direct plating were shown to decline over 10 days storage as did the frequency of Campylobacter-positive carcasses determined by enrichment. Willis and Murray (20) were unable to isolate Campylobacter from

![Mean cfu Campylobacter or contaminants](image)

![FIGURE 4. Campylobacter recovery and enumeration (mean log CFU/ml) on four different agars from freshly processed broiler carcasses after enrichment in HEB or BEB enrichment broths.](image)

![FIGURE 5. Campylobacter recovery and enumeration (mean CFU/ml) from freshly processed broiler carcasses (n = 36) by direct plating onto three selective media.](image)

### TABLE 2. Recovery of Campylobacter on individual agars or combinations of agars by direct plating of 0.1-ml duplicate samples from 36 freshly processed broiler carcasses

<table>
<thead>
<tr>
<th></th>
<th>Cefex</th>
<th>mCCDA</th>
<th>MHBA</th>
<th>Cefex + mCCDA</th>
<th>Cefex + MHBA</th>
<th>mCCDA + MHBA</th>
<th>All three agars</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. positive/36</td>
<td>24</td>
<td>23</td>
<td>19</td>
<td>30</td>
<td>31</td>
<td>29</td>
<td>34</td>
</tr>
<tr>
<td>% positive</td>
<td>66.7</td>
<td>63.9</td>
<td>52.8</td>
<td>83.3</td>
<td>86.1</td>
<td>80.5</td>
<td>94.4</td>
</tr>
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</table>
retail-level market broiler chickens by the direct plating procedure even though 69.4% of the carcasses they tested were found to be positive for *C. jejuni* after enrichment in HEB.

In the current study, the direct plating method provided a simple, less expensive, more rapid alternative to traditional MPN procedures for estimating *Campylobacter* populations associated with freshly processed (postchill, post-drip) broiler carcasses. *Campylobacter* populations observed on these samples were in the same range as those reported in previous surveys (14–16, 19). HEB appeared to be a more sensitive selective enrichment broth than BEB for this type of sample, perhaps due to the microaerobic conditions employed by the HEB. Use of BEB under microaerobic conditions could improve recovery and will be considered in future trials. Recovery of *Campylobacter* was similar among the agars tested. Typical *Campylobacter* colonies may be enumerated by trained personnel on all of the agars; however, fewer contaminants observed on the MHBA facilitated *Campylobacter* isolation and enumeration. Both MHBA and Campy-cefex agar facilitate differentiation of translucent *Campylobacter* colonies because they are less opaque media than MCCDA. The MCCDA medium is very dark, and consequently, the translucent quality of *Campylobacter* colonies cannot be used to enumerate *Campylobacter* on this medium. MHBA was by far the most expensive of the agars, while Campy-cefex was the least expensive. Agar plates in these experiments were inoculated with 0.1 ml of sample. Plating 1.0-ml aliquots from the original carcass rinse samples (equally distributed across four agar plates) could increase the sensitivity of the direct plating procedure and will be considered in future trials.

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**REFERENCES**


