Isolation of Campylobacter from Brazilian broiler flocks using different culturing procedures

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ABSTRACT Conventional culturing methods enable the detection of Campylobacter in broiler flocks. However, laboratory culture of Campylobacter is laborious because of its fastidious behavior and the presence of competing nontarget bacteria. This study evaluated different protocols to isolate Campylobacter from broiler litter, feces, and cloacal and drag swabs. Samples taken from commercial Brazilian broiler flocks were directly streaked onto Preston agar (PA), Campy-Line agar (CLA), and modified charcoal cefoperazone deoxycholate agar (mCCDA) and also enriched in blood-free Bolton broth (bfBB) for 24 and 48 h followed by plating onto the different selective media. Higher numbers of Campylobacter-positive cloacal and drag swab samples were observed using either direct plating or enrichment for 24 h before plating onto PA, compared with enrichment for 48 h (P < 0.05). Furthermore, direct plating was a more sensitive method to detect Campylobacter in broiler litter and feces samples. Analysis of directly plated samples revealed that higher Campylobacter levels were detected in feces streaked onto PA (88.8%), cloacal swabs plated onto mCCDA (72.2%), drag swabs streaked onto CLA or mCCDA (69.4%), and litter samples inoculated onto PA (63.8%). Preston agar was the best agar to isolate Campylobacter from directly plated litter samples (P < 0.05), but there was no difference in the efficacies of PA, mCCDA, and CLA in detecting Campylobacter in other samples. The isolated Campylobacter strains were phenotypically identified as Campylobacter jejuni or Campylobacter coli. The predominant contaminant observed in the Campylobacter cultures was Proteus mirabilis, which was resistant to the majority of antimicrobial agents in selective media. Together, these data showed that direct plating onto PA and onto either CLA or mCCDA as the second selective agar enabled the reliable isolation of thermophilic Campylobacter species from broiler samples. Finally, Campylobacter was detected in all broiler flocks sampled.

Key words: Campylobacter jejuni, Campylobacter coli, selective culture, Proteus mirabilis, food safety

INTRODUCTION

Campylobacteriosis is a leading global public health issue and is the third most common foodborne disease in the European Union (EFSA, 2013). After a significant decline, the number of human Campylobacter infections in the United States increased in 2012, when the incidence was 14.30 cases per 100,000 inhabitants (CDC, 2013). Campylobacter jejuni currently accounts for 90% of the cases of foodborne campylobacteriosis confirmed in the laboratory, followed by C. coli, which causes 8% of the cases (CDC, 2013). Campylobacter exposure includes contact with infected animals and consumption of either untreated water or contaminated foods of animal origin, such as raw milk and meat (EFSA, 2013). Because of the optimal growth temperature and colonization ability, thermophilic Campylobacter species have effectively adapted to the avian gut, where they are commonly found in high numbers (Lee and Newell, 2006; Hermans et al., 2012). Although Campylobacter colonization of the broiler gut is not related to clinical disease, it might subsequently lead to contamination of broiler meat during processing (Rosenquist et al., 2006; Hermans et al., 2012). Carcasses generally become contaminated during defeathering or by the leakage of feces from colonized broilers due to eventual gut rupture during evisceration (Franchin et al., 2005; Reich et al., 2008). Several studies have reported the high mean prevalence of thermophilic Campylobacter in broiler carcasses (Rosenquist et al., 2006; Kuana et al., 2008; EFSA, 2013). Therefore, handling or consumption of undercooked chicken is considered a major source of human campylobacteriosis (EFSA, 2013).

Campylobacter control in broilers is a multifactorial process and involves all steps of the food chain including primary production (Rosenquist et al., 2006; Reich et al., 2008; Hermans et al., 2012). Therefore, it is important to establish surveillance programs in broiler...
farms to determine the *Campylobacter* status of flocks. Several selective broths and agars have proved efficient for the accurate isolation of *Campylobacter* from different samples such as chicken and other foods (Corry et al., 1995; Baylis et al., 2000; Line, 2001; Williams et al., 2009; Chon et al., 2012; Rodgers et al., 2012; Ugarte-Ruiz et al., 2012). However, *Campylobacter* is particularly sensitive to environmental stress, including variations in temperature, pH, osmolarity, atmospheric oxygen, and sunlight (Lee and Newell, 2006). Due to their fastidious behavior, *Campylobacter* strains grow slowly under laboratory conditions, and culturing is possible only under specific conditions. In selective media supplements such as blood, charcoal, ferrous sulfate, sodium metabisulfite, sodium pyruvate, and hemin can protect *Campylobacter* cells from the damage caused by oxygen derivatives (Corry et al., 1995). However, supplementation with antimicrobials also inhibits the growth of competing and less fastidious microorganisms (Ng et al., 1985; Moran et al., 2011).

Although direct plating onto selective media (Musgrove et al., 2001; Potturi-Venkata et al., 2007; Kiess et al., 2010; Rodgers et al., 2012) or prior enrichment in selective broth (Franchin et al., 2005; Williams et al., 2009, 2012) have been used to detect *Campylobacter* in samples taken from broiler flocks, there is no widely accepted protocol. Depending on the sample and transport conditions, low numbers of viable or stressed *Campylobacter* cells might require selective enrichment to grow to a detectable level (Hutchinson and Bolt, 1983; Ugarte-Ruiz et al., 2012). However, enrichment of more contaminated specimens such as fecal samples commonly yields lower levels of *Campylobacter*-positive samples (Musgrove et al., 2001; Kiess et al., 2010; Rodgers et al., 2010) and allows the spread of background flora (Musgrove et al., 2001; Alves et al., 2012). Furthermore, some *Campylobacter* species such as *C. coli* might be inhibited by selective supplements in the agar and broth (Ng et al., 1985; Rodgers et al., 2010; Williams et al., 2012). To monitor the contamination level in flocks, it is important to develop strategies to reliably isolate *Campylobacter* from broiler samples. Therefore, this study evaluated enrichment culturing and direct plating onto different selective media to isolate thermophilic *Campylobacter* from different samples taken from commercial broiler flocks in Brazil.

**MATERIALS AND METHODS**

**Broiler Samples**

In this study, 18 broiler flocks between 5 and 6 wk of age from 2 large-scale broiler production companies in Southern Brazil were sampled. A total of 36 cloacal swabs, 36 pooled feces, 36 drag swabs, and 36 pooled litter samples were collected from February 2011 to October 2011. Two samples of each material were taken from individual broiler flocks. Cloacal swabs from both sampled broilers were pooled and placed in screw-cap tubes containing Cary-Blair (Oxoid, Basingstoke, UK) transport media. Drag swabs were placed in a sterile plastic bag containing 150 mL of 1% buffered peptone water (BPW). Pooled broiler litter or fresh feces were added to 3 times the volume of 1% BPW to obtain a sample/diluent ratio of 1:4 (mass/volume). All samples were transported to the laboratory in insulated boxes with ice packs and processed immediately after arrival.

**Campylobacter Culture**

Two methods were evaluated: enrichment culturing and direct plating onto different selective media. Cloacal swabs were individually streaked onto 2 Preston agar plates (PA; Hutchinson and Bolton, 1983) supplemented with 5 IU/mL of polymyxin B, 10 μg/mL of rifampicin, 10 μg/mL of trimethoprim lactate, and 10 μg/mL of amphotericin B; Campy-Line agar plates (CLA; Line, 2001) contained 20 μg/mL of trimethoprim lactate, 20 μg/mL of vancomycin, 20 μg/mL of cefoperazone, and 50 μg/mL of cycloheximide; and modified charcoal cefoperazone deoxycholate agar plates (mCCDA; Hutchinson and Bolton, 1984) supplemented with 32 μg/mL of cefoperazone and 10 μg/mL of amphotericin B. Feces, drag swabs, and litter samples suspended in 1% BPW were mixed thoroughly and individually inoculated onto duplicated PA, CLA, and mCCDA plates. All plates were incubated at 41.5°C in microaerobic atmosphere (5% O2, 10% CO2, and 85% N2; White Martins, Rio de Janeiro, Brazil) for 44 h (±4 h). Next, cloacal swabs were inoculated in 15 mL of blood-free Bolton broth (bfBB; Williams et al., 2009) supplemented with 20 μg/mL of cefoperazone, 20 μg/mL of vancomycin, 20 μg/mL of trimethoprim lactate, and 10 μg/mL of amphotericin B. The 1% BPW suspensions of feces, broiler litter, and drag swabs were mixed thoroughly and 1 mL of suspended swabs was inoculated in 9 mL of bfBB, whereas 10 mL of each broiler litter and drag swab suspension was individually inoculated in 90 mL of bfBB. The samples were incubated at 41.5°C in microaerobic atmosphere. Selective enrichment was evaluated after 24 h (±4 h) and 48 h (±4 h) by streaking onto 2 PA, CLA, and mCCDA plates, which were then incubated at 41.5°C for 44 h (±4 h) in microaerobic atmosphere. The *C. jejuni* ssp. *jejuni* ATCC 33560 control culture was inoculated in sterile bfBB and streaked onto mCCDA, PA and CLA in parallel with each group of daily samples to ensure proper performance of the media during the course of sample analysis. Selective media and broth were purchased from Oxoid, whereas antimicrobials and other selective supplements were acquired from Sigma-Aldrich (St. Louis, MO).

**Campylobacter Identification**

A single typical or putative *Campylobacter* colony isolated from each plate of PA, CLA, and mCCDA after either direct plating or enriched culturing for 24 and
48 h was subcultured on blood agar no. 2 (Oxoid) and on Columbia agar (Merck, Darmstadt, Germany) and then incubated in microaerobic atmosphere at 41.5°C for 24 to 48 h (±4 h). Gram-negative colonies exhibiting curved or spiral rods were presumptively identified as *Campylobacter* and further tested for catalase, oxidase, hippurate hydrolysis, and indoxyl acetate hydrolysis. *Campylobacter jejuni* ssp. *jejuni* ATCC 33560, *C. coli* ATCC 35559, and *C. lari* ATCC 35221 were used as control strains.

**Characterization and Antimicrobial Resistance of Contaminants from Campylobacter Cultures**

A total of 24 non-*Campylobacter* strains that overgrew *Campylobacter* cultures on CLA (n = 15) and nCCDA (n = 9) plates were randomly selected for further analysis. A single atypical colony per selected plate was subcultured onto MacConkey agar (Merck) and brain heart infusion agar (Merck) and further analyzed by standard biochemical procedures (Holt et al., 1994). Next, the isolated non-*Campylobacter* strains were tested for their resistance to the antimicrobial agents present in the selective media used in this study. Antimicrobial susceptibility testing was performed using the Mueller-Hinton (Merck) broth microdilution method (CLSI, 2012) to determine minimum inhibitory concentrations (MIC) for trimethoprim lactate, cefoperazone, vancomycin, polymyxin B, and rifampicin (Sigma-Aldrich). The antimicrobial dilution ranges tested were as follows: 8,000 to 62.5 μg/mL for trimethoprim lactate, cefoperazone, vancomycin, and polymyxin B, and 80 to 0.6 μg/mL for rifampicin. The inoculated microdilution trays were sealed with plastic tape and incubated at 35 ± 2°C for 16 to 20 h. The MIC was defined as the lowest concentration of each antimicrobial at which there was complete growth inhibition of isolates. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as quality control strains.

**Statistical Analysis**

First, the effect of enrichment and selective media to isolate *Campylobacter* from different broiler samples was evaluated using Fisher’s exact test (SAS Institute Inc., 2008). Nested analyses were performed to evaluate individual effects as follows. Whenever a significant effect ($P < 0.05$) was observed, a detailed analysis comparing the 2 treatments was performed. Subsequently, Fisher’s exact test was used to analyze whether there were significant differences in the use of different selective media for the direct isolation of each *Campylobacter* species. A descriptive analysis was used to determine whether combining the data for the different selective agars yielded higher numbers of directly plated *Campylobacter*-positive samples.

**RESULTS**

**Comparison Between Culturing Procedures and Selective Media**

Table 1 shows the number of *Campylobacter*-positive broiler samples observed by direct plating and by enrichment followed by streaking onto selective agar. Regardless of the selective medium used, direct plating yielded higher numbers of *Campylobacter*-positive broiler litter and feces samples compared with selective enrichment ($P < 0.05$). The drag and cloacal swabs directly plated onto either CLA or mCCDA yielded higher numbers of positive samples than selective enrichment ($P < 0.05$), but there was no significant difference between direct plating onto PA and enrichment for 24 h before plating onto PA for these samples. Therefore, irrespective of the selective agar used, enrichment in bfBB for 48 h did not improve the isolation of *Campylobacter* from the broiler samples analyzed. Notably, samples enriched for either 24 or 48 h revealed an abundant proliferation of non-*Campylobacter* cells on mCCDA and CLA, which overgrew the culturing plates (data not shown).

When the samples were directly plated, there was no difference among the selective media used to isolate *Campylobacter* from broiler samples; however, a higher number of *Campylobacter*-positive litter samples was observed using PA ($P < 0.05$). Using direct culturing, the highest levels of thermophilic *Campylobacter* were observed with feces plated onto PA (88.8%), cloacal swabs streaked on mCCDA (72.2%), drag swabs plated on either CLA or mCCDA (69.4%), and litter
samples inoculated onto PA (63.8%; Table 1). The descriptive analysis of the combination of the data for the different selective agars indicated increased likelihood of detecting Campylobacter-positive broiler samples (Table 2).

**Detection of Thermophilic Campylobacter Species in Broiler Samples**

Thermophilic Campylobacter strains isolated from broiler samples using direct plating and enriched cultures were characterized as *C. jejuni* or *C. coli* using phenotypic analyses. Analysis of directly plated samples revealed that the highest rate of *C. jejuni* isolation was observed in fresh feces cultured onto PA (86.1%), whereas *C. coli* was identified more often in feces plated onto CLA (25.0%; Table 3). Therefore, the isolation of Campylobacter species from the samples varied depending on the selective medium used. Campylobacter *jejuni* strains were isolated more often from broiler litter samples directly plated onto PA than either CLA or mCCDA (*P* < 0.05). Direct plating onto PA also yielded more *C. jejuni*-positive feces samples than plating onto CLA (*P* < 0.05). However, higher numbers of *C. coli*-positive feces samples were observed by directly plating onto CLA than onto PA (*P* < 0.05), and direct plating onto mCCDA yielded more *C. coli*-positive samples, compared with PA (*P* < 0.05).

**Competing Microorganisms in Campylobacter Cultures**

All the subcultured non-Campylobacter strains were phenotypically characterized as *Proteus mirabilis*. The MIC of cefoperazone, trimethoprim lactate, polymyxin B, and vancomycin revealed that addition of these antimicrobial agents to the broth and selective media did not prevent the growth of *P. mirabilis* strains (Table 4). However, the MIC of rifampicin for majority of the *P. mirabilis* strains analyzed (21/24) was below the concentration of rifampicin used in PA (10 μg/mL; data not shown).

**DISCUSSION**

Direct plating has been used to detect *Campylobacter* in highly contaminated broiler samples (Musgrove et al., 2001; Kiess et al., 2010; Rodgers et al., 2010; Ugarte-Ruiz et al., 2012). In this study, direct plating onto PA was the most sensitive method to isolate *Campylobacter* from broiler litter samples (*P* < 0.05); however, for fecal and cloacal and drag swab samples there was no difference among PA, mCCDA, and CLA in the ability to detect *Campylobacter* (Table 1). Comparison of selective agars used for culture of directly plated fecal broiler samples has shown that mCCDA is the most sensitive agar to isolate *Campylobacter* (Oakley et al., 2012; Rodgers et al., 2012), whereas CLA yields fewer *Campylobacter*-positive samples (Potturi-Venkata et al., 2007).

Selective enrichment does not improve *Campylobacter* detection in broiler litter samples (Kiess et al., 2010) and yields fewer *Campylobacter*-positive cecal samples than direct plating onto selective media (Musgrove et al., 2001). This study showed that selective enrichment in bfBB did not improve the recovery of *Campylobacter* from broiler samples evaluated (Table 1). With the exception of cloacal and drag swabs enriched for 24 h before plating onto PA, the lower rate of *Campylobacter*-positive enriched samples observed cannot be attributed to the absence of blood in Bolton broth. The absence of blood in Bolton broth has no detrimental effect on the growth of *Campylobacter* from broiler fecal samples (Williams et al., 2009). Other studies have used bfBB enrichment for the detection of *Campylobacter* in chicken samples because other components provide protection against toxic oxygen derivatives (Paulsen et al., 2005; Chon et al., 2012), whereas the use of bfBB also enables downstream PCR analyses of the enriched cultures (Bolton et al., 2002; Jøsefsen et al., 2004).

Samples enriched in bfBB and plated onto mCCDA or CLA showed extensive spreading of contaminant bacteria. This overgrowth of competing flora on selective agar frequently hampers the recognition of *Campylobacter* colonies (Musgrove et al., 2001; Kiess et al., 2010; Moran et al., 2011; Alves et al., 2012). Although *E. coli* and *Pseudomonas* spp. are the con-
taminant microorganisms most frequently recovered from cultures enriched in different Campylobacter selective broths (Baylis et al., 2000), this study revealed that P. mirabilis was the main competitor bacterium in Campylobacter cultures. Comparison of 16S rRNA tagged-pyrosequencing analysis of broiler feces with sequences from bacterial colonies taken from different Campylobacter selective media has shown that the background flora belong to few genera in which Proteus was the most abundant (Oakley et al., 2012). The isolated P. mirabilis strains were resistant to cefoperazone, trimethoprim lactate, polymyxin B, and vancomycin at the concentrations used in the broth and selective agar, which revealed reduced selectivity (Table 4). Addition of vancomycin to selective media inhibits the growth of competing gram-positive bacteria (Corry et al., 1995). However, the concentration of rifampicin used was sufficient to inhibit the growth of majority of the P. mirabilis strains on PA (Table 4). This is consistent with the results of Chon et al. (2012) who observed that PA limits the growth of competing bacteria in enriched Campylobacter cultures from chicken samples, compared with other selective agars such as mCCDA. Notably, the cloacal and drag swabs enriched in bfBB for 24 h and plated onto PA yielded higher numbers of Campylobacter-positive samples than enriched cultures plated onto CLA or mCCDA (P < 0.05; Table 1). The recovery of Campylobacter from enriched cultures might be influenced by the antimicrobial composition of the subsequent plating agar (Ugarte-Ruiz et al., 2012).

Phenotypic characterization of thermophilic Campylobacter strains revealed that C. jejuni was the most common species isolated from broiler samples (Table 3). This observation is consistent with the higher prevalence of C. jejuni in broiler flocks (Franchin et al., 2005; Potturi-Venkata et al., 2007; Reich et al., 2008; Kuana et al., 2008). These data highlight the relevance of this species for public health because it is commonly associated with human campylobacteriosis (CDC, 2013; EFSA, 2013). Notably, samples might contain multiple Campylobacter species, suggesting mixed colonization (Potturi-Venkata et al., 2007; Ugarte-Ruiz et al., 2012). Direct plating analyses revealed that broiler feces streaked onto CLA and drag swabs plated onto mCCDA yielded higher numbers of C. coli-positive samples compared with plating onto PA (P < 0.05; Table 3). The lower detection of C. coli on PA might be due to the restrictive effect of antimicrobials such as rifampicin (Ng et al., 1985). The PA is less effective than other selective media for the direct isolation of Campylobacter strains from cecal contents (Rodgers et al., 2010); however, this study shows that PA is highly selective for C. jejuni (Table 3). These data suggest that it is important to use at least 2 selective agars with different antimicrobial compositions. The use of 2 or 3 selective agars was more effective than one agar for the detection of Campylobacter in broiler samples (Table 2).

In this study, all Brazilian broiler flocks sampled at preharvest age tested positive for Campylobacter in at least one of the culturing techniques. High numbers of Campylobacter-positive flocks were detected in previous studies (Franchin et al., 2005; Kuana et al., 2008; Reich et al., 2008; Williams et al., 2012). The Campylobacter status of broiler flocks has an effect on the number of Campylobacter-positive carcasses at processing (Rosenquist et al., 2006; Reich et al., 2008); however, current strategies are not completely effective for preventing the colonization of broilers in farms (Hermans et al., 2012). This study showed that direct culturing significantly increased the isolation of thermophilic Campylobacter from feces and broiler litter samples compared with enrichment in bfBB for 24 or 48 h. The direct culturing method was also effective for detecting thermophilic Campylobacter in drag and cloacal swabs. Direct plating onto PA and onto either CLA or mCCDA as the second selective agar reduced the time required for Campylobacter detection and enabled the isolation

### Table 3. Campylobacter species (%) isolated by directly plating broiler samples onto selective media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Litter</th>
<th>Cloacal swabs</th>
<th>Feces</th>
<th>Drag swabs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Campylobacter jejuni</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA^1</td>
<td>23/36 (63.8)^a</td>
<td>21/36 (58.3)</td>
<td>31/36 (86.1)^a</td>
<td>22/36 (61.1)</td>
</tr>
<tr>
<td>CLA^2</td>
<td>8/36 (22.2)^b</td>
<td>23/36 (63.8)</td>
<td>19/36 (52.7)^b</td>
<td>21/36 (58.3)</td>
</tr>
<tr>
<td>mCCDA^3</td>
<td>7/36 (19.4)^b</td>
<td>22/36 (61.1)</td>
<td>25/36 (69.4)^ab</td>
<td>19/36 (52.7)</td>
</tr>
<tr>
<td><strong>Campylobacter coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>0/36 (0.0)</td>
<td>1/36 (2.7)</td>
<td>1/36 (2.7)^b</td>
<td>0/36 (0.0)^b</td>
</tr>
<tr>
<td>CLA</td>
<td>1/36 (2.7)</td>
<td>1/36 (2.7)</td>
<td>9/36 (25.0)^a</td>
<td>4/36 (11.1)^ab</td>
</tr>
<tr>
<td>mCCDA</td>
<td>1/36 (2.7)</td>
<td>3/36 (8.3)</td>
<td>5/36 (13.8)^ab</td>
<td>6/36 (16.6)^a</td>
</tr>
</tbody>
</table>

^a,bValues followed by different superscripts in the same column differ significantly (P < 0.05).

1Preston agar.
2Campy-Line agar.
3Modified charcoal cefoperazone deoxycholate agar.

### Table 4. Minimum inhibitory concentration (MIC) ranges observed using Proteus mirabilis strains isolated from Campylobacter cultures

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lowest range</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>250</td>
</tr>
<tr>
<td>Trimethoprim lactate</td>
<td>2,000</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>&gt;8,000</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>2</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>4,000</td>
</tr>
</tbody>
</table>
of thermophilic Campylobacter species with different growth requirements. Finally, the high rate of culture-positive Brazilian broiler flocks observed at preharvest age highlights the requirement for feasible intervention measures to reduce the intestinal colonization of broilers by Campylobacter.

ACKNOWLEDGMENTS

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CDC (Centers for Disease Control and Prevention). 2013. Incidence measures to reduce the intestinal colonization of broiler age highlights the requirement for feasible intervention positive Brazilian broiler flocks observed at preharvest growth requirements. Finally, the high rate of culture- Campylobacter of thermophilic Campylobacter jejuni in broiler caecal contents using culture-based methods. J. Appl. Microbiol. 109:1244-1252.


