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Campylobacter in broiler slaughter samples assessed by direct count on mCCDA and Campy-Cefex agar

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ABSTRACT

Campylobacter spp. cause foodborne illnesses in humans primarily through the consumption of contaminated chicken. The aim of this study was to evaluate the United States Department of Agriculture's (USDA) recommended methodology, protocol MLG 41.02, for the isolation, identification and direct plate counting of *Campylobacter jejuni* and *C. coli* samples from the broiler slaughtering process. A plating method using both mCCDA and Campy-Cefex agars is recommended to recover *Campylobacter* cells. It is also possible to use this method in different matrices (cloacal swabs and water samples). Cloacal swabs, samples from pre-chiller and post-chiller carcasses and samples of pre-chiller, chiller and direct supply water were collected each week for four weeks from the same flock at a slaughterhouse located in an abattoir in southern Brazil. Samples were analyzed to directly count *Campylobacter* spp., and the results showed a high frequency of *Campylobacter* spp. on Campy-Cefex agar. For the isolated species, 72% were identified as *Campylobacter jejuni* and 38% as *Campylobacter coli*. It was possible to count *Campylobacter jejuni* and *Campylobacter coli* from different samples, including the water supply samples, using the two-agar method. These results suggest that slaughterhouses can use direct counting methods with both agars and different matrices as a monitoring tool to assess the presence of *Campylobacter* bacteria in their products.

Introduction

Campylobacter bacteria are a major cause of foodborne illness in humans and are the most common gastroenteritis-causing bacteria in the world. In both developed and developing countries, they cause more cases of gastroenteritis than does foodborne *Salmonella*. The high incidence of *Campylobacter* diarrhea and its duration and possible sequelae make it highly important from a socio-economic perspective. In developing

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countries, *Campylobacter* infections in children under the age of two are particularly frequent and occasionally result in death.[1]

In the USA, *Campylobacter* is the second most isolated agent of foodborne illness,[2] and in the European Union (EU), *Campylobacter* is the main pathogen that causes human gastroenteritis,[3] with approximately 198,252 cases in 2009 alone.[4]

Campylobacter bacteria can be spread by contaminated food and water, and chicken was implicated as the main contamination source.[5] During the poultry slaughtering process, carcass contamination can occur not only by high bacterial loads present in the poultry's gastrointestinal tract but also by the bacterial loads present in skin and feathers.[6] In addition to initial flock contamination, hygienic and sanitary conditions during both the slaughtering process and carcass conservation can influence the presence and level of *Campylobacter* in the final product.[7]

Broiler carcasses contaminated with *Campylobacter* have been detected in many countries. A study conducted in Brazil[8] showed that 95 of the 96 broiler carcasses examined tested positive for *Campylobacter* at the end of the slaughter line. According to the World Health Organization,[9] reducing the prevalence or concentration at a specified point in the production chain has the potential to reduce the risk of human incidences if intervention is taken.

Quantitative microbial risk assessment is a well-recognized component of modern risk analysis and is used to estimate the impact of a particular hazard/product combination and/or changes in processing on public health. In this regard, two methods for *Campylobacter* quantification were published by leading authorities, one by the International Standard Organization, ISO-TS 10272-2,[10] and another by the United States Department of Agriculture, MLG 41.02.[11] The latter describes a method for direct plating and qualitative and quantitative evaluations using Campy-Cefex agar for the isolation, identification and counting of *Campylobacter* spp. present in rinsed poultry carcasses, sponges and raw product samples.

The present study aimed to test the MLG 41.02[11] methodology on carcass samples and in alternative matrices, such as cloacal swabs and water samples, from the broiler slaughtering process to compare the number of recovered *Campylobacter* cells on Campy-Cefex and mCCDA agar plates.

Materials and methods

Sampling

Samples were taken once a week from a slaughterhouse located in southern Brazil for four weeks in April 2013. Each time, three cloacal swabs and three samples from pre-chiller carcasses, post-chiller carcasses, pre chiller water, chiller water and direct supply water were collected, with 12 samples of each type and 72 in total. The samples were placed immediately into thermic boxes with ice and sent to the laboratory for microbiological analysis.

Quantitative and qualitative analysis

Controls

Campylobacter jejuni ATCC 33291 and *Campylobacter coli* ATCC 43578 were used as positive controls for un-inoculated agar plates and broth sample sets. One colony from each positive control sample was confirmed.

Carcass rinse procedure

Carcass samples were collected prior to placement in the pre-chiller tank on the evisceration line (pre-chiller samples) and immediately after the post-chiller (post-chiller samples).

To perform quantitative analysis on the carcass rinse samples, carcasses were put into sterile plastic bags with 400 mL of 1% BPW (1% Buffered Peptone Water, Oxoid®, Basingstoke, Hampshire, UK) and mixed thoroughly by gently shaking for 3 min. From the rinse solution, 250 μL was streaked onto four Campy-Cefex agar plates with antimicrobial selective supplement (SR0155, Oxoid®, Basingstoke, Hampshire, UK) and 5% sterile laked equine blood (Ebefarma®, Cachoeira de Macacu, RJ, Brazil) using sterile glass Drigalski loops. Another 100 μL from the rinse solution was streaked onto two Campy-Cefex agar plates. The procedure was performed in duplicate with streaking onto mCCDA agar plates containing an antimicrobial selective supplement (SR0155 Oxoid®, Basingstoke, Hampshire, UK). The agar plates were incubated at 42 ± 1.0 °C for 48 ± 2 h under microaerobic conditions (Microerobac, Probac®, São Paulo, SP, Brazil).

To perform qualitative analysis, 30 mL of the rinsing solution was added to 30 mL of double strength blood-free Bolton enrichment broth (2X BF-BEB, Oxoid®, Basingstoke, Hampshire, UK) with selective supplement (SRE183, Oxoid® Basingstoke, Hampshire, UK) and homogenized as described above. After incubation, 10 μL from the BF-BEB was streaked onto Campy-Cefex and mCCDA agar plates and incubated under microaerobic conditions at 42 ± 1.0 °C for 48 ± 2 h.

Cloacal swabs

Swabs were collected during broiler hanging, one swab per bird, and stored in a 10 mL flask with 1% BPW (Oxoid® Basingstoke, Hampshire, UK) at 4 °C. The tubes were homogenized, and the quantitative analysis followed the same methodology as described above for the carcasses. To perform the qualitative analysis, 3 mL from the BPW homogenized solution was added to 30 mL of double strength blood-free Bolton enrichment broth (2X BF-BEB, Oxoid®, Basingstoke, Hampshire, UK) supplemented with a selective supplement (SRE183, Oxoid®, Basingstoke, Hampshire, UK) and analyzed as described above for the carcasses.

Water samples

Water samples from the pre chiller, chiller and direct water supply were collected in 50 mL sterile flasks and stored at 4 °C in the laboratory. The water samples were homogenized and quantitative and qualitative analysis was performed as described above for the carcasses.

Table 1 – Frequency of *Campylobacter* spp. colonies directly isolated from mCCDA and Campy-Cefex agar plates with or without broth enrichment in different samples from the broiler slaughtering process.

Sample	Direct isolation Agar Campy-Cefex (%)	Direct isolation Agar mCCDA (%)	Enrichment broth and agar isolation* (%)	Total of analyzed samples
Swabs	100	100	100	12
Carcasses pre-chiller	100	25	100	12
Pre-chiller water	100	75	100	12
Chiller water	100	66	100	12
Carcasses post-chiller	83	67	100	12
Water supply	100	50	100	12

* Bolton broth, Campy-Cefex and mCCDA agars.

Plate analysis and results

After incubation, all typical *Campylobacter* colonies were counted if the total number of cells fell within a range of 15–300 colonies. The interpretation of typical colonies followed the instructions in the MLG 41.02 protocol.[11] Briefly, if the four plates with 250 μL were countable, the sum of the counts from the four plates was determined. If the only countable plates were the two plates with 100 μL of sample, the total number of cells on both plates was averaged and multiplied by ten. If both dilutions were within the countable interval, the final count was determined by averaging the results calculated above. All cell count results are represented as CFU/mL. If the final count was >300 CFU in each of the six plates, "TNTC" (to numerous to count) was recorded, or an estimated counting of >2100 CFU/mL was used.[11]

Examination and confirmation of colonies

A typical colony from each sample was picked to confirm and test motility, morphology, oxidase and catalase activity (Probac®, São Paulo, SP, Brazil) and latex agglutination (Dryspot, DR0150, Oxoid®, Basingstoke, Hampshire, UK), as described in MLG 41.02.[11] These colonies were also used for the polymerase chain reaction (PCR) technique (see below).

PCR methodology

Multiplex PCR was performed as described by Perdoncini et al.[12] to identify *C. jejuni* and *C. coli*. Briefly, 30 μL reactions containing 10× buffer, 1.5 mM $MgCl_2$, 5 mM dNTPs, two units of Taq polymerase, thermo DNA extract, 4 pmol/μL of each 16S rRNA primer, 2 pmol/μL specific primers and ultra-pure water up to 30 μL were made. The specific primers used amplify the following genes: *map*A (F^a–CTATTTTATTTTTGAGTGCTTGTG, R^b–GCTTTATTTGCCATTTGTTTTATTA with 589 pb, 50 N) (Invitrogen®, São Paulo, SP, Brazil) and *ceu*E (F^a–AATTGAAAATTGCTCCAACTATG, R^b–TGATTTTATTATTTGTAGCAGCG with 462 pb and a common region between species (16S rRNA), 50 N (Invitrogen®, São Paulo, SP, Brazil) and F^a–ATCTAATGGCTTAACCATTAAAC, R^b–GGACGGTAACTAGTTTAGTATT with 857 pb, 50 N (Invitrogen®, São Paulo, SP, Brazil)). Amplification reactions were carried out in a thermal cycler (Swift MaxPro®, Esco, Hatboro, PA, USA) under the following conditions: denaturation for 10 min at 95 °C, 35 cycles at 95 °C for 30 s, annealing at 59 °C for 1 min and 30 s and a final extension at 72 °C for 10 min. *Arcobacter* spp. were used as negative controls and *C. jejuni* ATCC 33291 and *C. coli* ATCC 43578 as positive controls. Ten microliter aliquots of the reaction mixtures were electrophoresed through 1.5% agarose gels (with the addition of 20% ethidium bromide) with a 100-bp DNA ladder (Invitrogen®, São Paulo, SP, Brazil) to determine the molecular weight. Fragments were transilluminated with UV light.

Data statistical analysis

Data were submitted for ANOVA analysis using BioStat Version 2009 (Analyst Soft. Inc., Alexandria, VA, USA).

Results

The *Campylobacter* direct plate count frequency was higher on the Campy-Cefex agar than on the mCCDA agar for different samples from broiler slaughtering process as described in Table 1.

Table 2 presents the results of quantitative analysis of *Campylobacter* spp. directly counted on Campy-Cefex and mCCDA agar plates.

There was a high frequency of *C. jejuni* in all PCR-analyzed samples (Table 3). In addition, 18% contained both *C. jejuni* and *C. coli* in the same sample.

With respect to all PCR samples, 2% contained none of the specific genes used to identify *C. jejuni* or *C. coli*, although they were identified as a *Campylobacter* species.

Discussion

Campylobacter spp. are recognized as a main cause of human enteritis outbreaks in both developed and developing countries. Although Brazil is the world's largest poultry meat exporter, data regarding this pathogen are limited, and at present, there is no legislation pertaining to *Campylobacter* risk analyses or control methods.

The *Campylobacter* isolation methodologies are laborious, and there are many broths and agars available. Some studies have evaluated the effectiveness of different broths and agar plates for their ability to isolate *Campylobacter* from several matrices to develop more efficient and lower cost methods.[13] Oyarzabal et al.[14] evaluated 240 samples of broiler carcass rinse samples by recovering *Campylobacter* on Campy-Cefex, mCCDA and CLA (Campy-Line agar) agar plates. The authors concluded that with regards to time, preparation, performance

Table 2 – Direct count averages of *Campylobacter* spp. from Campy-Cefex and mCCDA agar plates plated with different samples from the broiler slaughtering process.

Samples	Agar Campy-Cefex Average (CFU/mL)	Agar mCCDA Average (CFU/mL)	p^*
Swabs	1.3×10^3	9.5×10^2	0.307
Carcasses pre-chiller	9.8×10^2	8.3×10^1	0.005
Pre-chiller water	1.7×10^2	5.4×10^2	0.502
Chiller water	8.0×10^2	3.0×10^1	0.139
Carcasses post chiller	1.5×10^2	3.8×10^1	0.194
Water supply	7.3×10^1	4.7×10^0	0.318

* $p < 0.05$, statistically significant.

Table 3 – Percentage of isolated *Campylobacter jejuni* and *Campylobacter coli* on Campy-Cefex and mCCDA agar plates as identified by PCR analysis.

Samples	*Campylobacter jejuni*		*Campylobacter coli*	
	Campy-Cefex	mCCDA	Campy-Cefex	mCCDA
Swabs	12%	8%	10%	8%
Carcasses pre-chiller	8%	6%	–	2%
Pre-chiller water	6%	6%	–	–
Chiller water	6%	2%	2%	–
Carcasses post-chiller	4%	6%	–	6%
Water supply	4%	–	4%	–

and cost, Campy-Cefex and mCCDA agar obtained better *Campylobacter* counting results from carcass rinse samples. In another poultry study[15] was compared five agar plates that were used to isolate *Campylobacter* as of cecal and fecal samples obtained from 60 broiler chicken. The mCCA agar was more efficient at isolating the bacteria than the mCCDA, CLA, CAP (*Campylobacter agar plates*) and *Campylobacter* agars.

In the present study, the quantitative methods from the MLG 41.02[11] protocol showed reduced levels of *Campylobacter* in samples collected along the slaughter line, from cloacal swabs from live birds to post chiller carcasses, suggesting that the process of slaughtering can have a beneficial effect on the microbiological status of carcasses at the end of the slaughtering line. Accordingly, Berrang et al.,[16] reported that improved hygiene in the slaughtering process and constant evaluations of such hygiene measures allowed for a reduction in *Campylobacter* spp. numbers in carcasses prior to shipping to markets. Thus, the number of bacteria from infected flocks can be reduced during processing in the slaughterhouse.

The presence of *Campylobacter* in the water supply samples (Table 1) was not expected because chemical treatment of the water should eliminate bacteria; further, if bacteria were present, a low level should not have been detected by a direct count method. It is known that *Campylobacter* is able to compose biofilms and that their formation has been proposed as a survival mechanism outside the host for protection against chemical products, physical cleaning processes and environmental stress, among others.[17] Thus, the presence of biofilms and the contamination of the water sources are possible explanations for these data.

A high (100%) prevalence of *Campylobacter* in cloacal swabs was also found in this study (Table 1) by direct counting on both types of agar. These data are in agreement with another study,[18] who found that 96.6% from 30 samples of cloacal swabs contained *Campylobacter*. Additionally, Evans and Sayers[19] identified these bacteria in 91% of chicken cloacal swabs (20 total samples) in Great Britain, and Franchin et al.[20] reported that 75% of the swabs from broiler flocks in southern Brazil were positive for these bacteria.

Regarding carcass contamination, we found *Campylobacter* contamination in 83% of post-chiller carcasses, and the isolation frequency by direct plate counting on Campy-Cefex agar was high (Table 1). After enrichment in Bolton broth to boost low cell numbers in some samples, all pre- and post-chiller carcass samples tested positive. However, these data were higher than those reported by the Europeans, who had an average poultry carcass contamination level of 75.8%,[21] and by Kuana et al.,[8] who reported that 98.3% of 60 broiler carcasses were contaminated after chiller processing. In the present work, a significant difference was found between the Campy-Cefex and mCCDA plates used for cell recovery in the analysis of pre-chiller carcass samples, where Campy-Cefex had higher *Campylobacter* cell numbers (Table 2). Furthermore, it is important to note that despite the high isolation percentage from the Campy-Cefex agar, no *C. coli* was recovered in pre- and post-chiller carcass samples, a fact that is not consistent with other matrices (Table 3).

Studies using direct plate counting methods have indicated that selective enrichment does not increase the recovery of *Campylobacter* from fecal or cecal samples or chicken carcasses.[22,23] These data differ from the present study where 100% of enriched samples were positive for *Campylobacter* spp. Kiess et al.[24] demonstrated that direct plating serves an advantage in isolating *Campylobacter* from poultry litter samples; 37% of the samples were positive for *Campylobacter* as tested by direct plating and 2% were positive following enrichment. Despite our higher *Campylobacter* frequency in enriched post-chiller samples, the direct count method was able to

recover and quantify *Campylobacter* in agreement with Oyarzabal et al.,[14] who demonstrated the value of direct plating in studying *Campylobacter* spp. contamination of poultry carcasses.

Multiplex PCR analysis identified 72% of samples as positive for *C. jejuni* and 38% as positive for *C. coli* (Table 3). Similar results were demonstrated in the European Union, where 60.8% of cecal samples tested positive for *C. jejuni* and 41.5% tested positive for *C. coli*.[21] In southern Brazil, a study conducted by Perdoncini et al.[12] sampled eight different points from a broiler slaughterhouse line. They identified *C. jejuni* in 75% of the samples and *C. coli* in 10% of the samples. In addition, both *C. jejuni* and *C coli* were present together in 15% of all samples. In contrast, 200 samples of broiler cecal content from a southern Brazil slaughterhouse were evaluated, and it was found that 44% were positive for *C. coli* and 2% were positive for *C. jejuni*.[25]

Studies have also been performed with regards to the different *Campylobacter* serotypes colonizing birds. Shibiny-El et al.[26] reported that it is not common to isolate more than one type or subtype of *Campylobacter* from the same bird. The authors suggest that *C. jejuni* and *C. coli* compete equally and showed a decline in *C. jejuni* and *C. coli* dominance in isolates from 35-day-old birds. In contrast, the present study, as well as the study by Perdoncini et al.,[12] isolated two different serotypes from the same sample; 18% of the samples contained both *C. jejuni* and *C. coli*. In fact, both serotypes appeared in the same frequency in cloacal swabs.

In this work, the ability to isolate *C. coli* from different matrices by Campy-Cefex and mCCDA agar plates was variable (Table 3). According to WHO,[1] many different forms of media can be used in the recovery of *Campylobacter* spp., and although mCCDA agar is the recommended medium, alternatives may be used. The main difference between media is the degree to which each inhibits contaminating flora, but all selective agents allow for the growth of both *C. jejuni* and *C. coli*.

Conclusions

The direct plating methods applied in this study were able to recover *Campylobacter* from different poultry matrices. Only from pre-chiller water did the Campy-Cefex agar direct counting method recover statistically high *Campylobacter* cells numbers. Based on the results of this study, it is plausible to suggest that both Campy-Cefex and mCCDA agar plates can increase the chances of recovering *C. coli* from swabs, carcasses and water samples. The present work also demonstrated that direct counting of *Campylobacter* from samples at different sites in the broiler slaughterhouse is useful for identifying contamination points and levels and is a possible tool for controlling *Campylobacter* contamination at Brazilian slaughterhouses.

Conflicts of interest

The authors declare no conflicts of interest.

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