Viability of *Campylobacter* spp. in frozen and chilled broiler carcasses according to real-time PCR with propidium monoazide pretreatment

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ABSTRACT The aim of this study was to evaluate the viability of *Campylobacter* spp. in frozen and chilled broiler carcasses using real-time PCR with propidium monoazide (PMA) pretreatment. Sixty broiler carcasses were collected: 30 frozen and 30 chilled. Each carcass was submitted to 2 real-time PCR protocols to detect and quantify *Campylobacter* spp.: one using pretreatment with PMA, which blocks the amplification of DNA from dead bacteria, and the other without PMA. The results showed that PMA-pretreated carcasses, either frozen or chilled, had a lower positivity rate compared to untreated samples ($P < 0.001$). Regarding storage temperatures, PMA-pretreated frozen carcasses that tested positive were in a lesser number than chilled carcasses ($P < 0.05$). However, the quantification of total and live bacteria in PMA-pretreated frozen carcasses that tested positive showed no significant difference compared to chilled carcasses. It was concluded that the real-time PCR with PMA pretreatment was a sensitive method for evaluating the viability of *Campylobacter* spp. in broiler carcasses. Chilled broiler carcasses would represent greater hazard to public health concerning *Campylobacter* transmission.

Key words: chicken meat, foodborne disease, DNA intercalator, campylobacteriosis

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INTRODUCTION

Infection by *Campylobacter* spp. is the most commonly reported food-borne disease in the world, along with salmonellosis, being the chicken meat the major vehicle associated with outbreaks of infection (Jorgensen et al., 2002; FAO/WHO, 2009; Hue et al., 2010). *Campylobacter jejuni* and *Campylobacter coli* are the main pathogens involved in human campylobacteriosis and are considered commensal organism in the poultry gut (Arsenault et al., 2007; Fitzgerald, 2015; Kaakoush et al., 2015). To avoid the contamination and proliferation of microorganisms in food, preservation methods, such as cooling and freezing, are widely used, both at the industrial and domestic levels (El-Shibiny et al., 2009; Sampers et al., 2010).

The gold standard methodology for the detection of *Campylobacter* spp. in chicken carcasses is the isolation and culture of the agent (Barros-Velazquez et al., 1999). However, analysis by this method is time-consuming and requires several days for the species confirmation. In addition, *Campylobacter* spp. under stressful conditions, such as those during refrigeration and freezing, may assume a viable but non-cultivable (**VBNC**) form, which cannot be grown, even in selective media, but retains its infectious ability and virulence (Barros-Velazquez et al., 1999; Donnison, 2003). Hence, molecular biological assays have been developed to improve the detection of these microorganisms in food samples and to reduce the time needed for the diagnosis (Oliveira et al., 2005; Schnider et al., 2010; Kruger et al., 2014). Nonetheless, it is important to note that polymerase chain reaction (**PCR**) by itself is not able to differentiate the DNA from dead bacteria from the DNA of live cells.

The differentiation between viable and VBNC cells from non-viable bacteria is a critical point in the assessment of contamination of broiler carcass by *Campylobacter* spp., because only live cells represent a real hazard to public health. Propidium monoazide (**PMA**), a derivative of propidium iodide, is able to covalently bind to double-stranded DNA from dead or injured cells, preventing DNA from being amplified, thereby

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enabling differentiation between viable and VBNC microorganisms from non-viable ones (Nocker et al., 2006).

Therefore, the aim of this study was to evaluate the viability of *Campylobacter* spp. in frozen and chilled broiler carcasses by real-time PCR with PMA.

MATERIALS AND METHODS

Sampling

The broiler carcasses tested were obtained from a poultry slaughterhouse under official veterinary inspection located in the Minas Gerais State, Brazil. Sixty broiler carcasses were collected: 30 frozen and 30 chilled. At each production shift, 2 carcasses were collected and identified after the chiller tank, one being directed to the chill chambers (chilled sample, 4°C ± 2°C), while the other was taken to the freezing tunnels (frozen sample, −20°C ± 4°C). Each broiler carcass was considered as a sample. The frozen samples were kept at refrigeration temperature (4°C to 8°C) for a period of 20 to 24 h before the analysis.

Positive Control

For all analyses, DNA from *Campylobacter jejuni* subsp. *jejuni* NCTC 11351^T cultured on Columbia blood agar (Himedia, Mumbai, India) supplemented with 5% defibrinated sheep blood for 48 h at 42°C under microaerobic atmosphere (5% O_2, 5% H_2, 10% CO_2, and 80% N_2) was used (ISO 10272-1/2006).

Preparation of the Broiler Samples

From each sample pieces of meat and skin from the thigh, wing, chest, cloacal region, ribs, and neck were collected. These pieces were cut into smaller ones and weighed on an analytical balance (Shimadzu AY220, Barueri, SP, Brazil) until complete 10 g (ISO 10272-1/2006). Then, the samples were placed in a sterile stomacher bag, and 90 mL of sterile PBS (0.01 M, pH 7.2) was added. Following, the bags were homogenized for 5 min in an automatic homogenizer (ITR MK 1204, Esteio, RS, Brazil). Two 1.5 mL aliquots of this mixture were placed in sterile microtubes, being one of the aliquots treated with PMA. Both aliquots were centrifuged (Thermo Scientific, Heraeus Megafuge 16R, Waltham, MA) at 16,000 × *g* for 10 min at room temperature, the supernatant was discarded, and the obtained pellets were weighed on the analytical balance. The weight of the pellets was standardized: 0.025 to 0.040 g.

PMA Treatment

Treatment of the samples with PMA (Biotium Inc., Fremont, CA) was performed according to Kruger et al. (2014) and to the manufacturer's instructions. PMA was stored in stock solutions of 10 mM in 20% DMSO at −20°C. The pellets obtained after the preparation of the samples were resuspended in 1 mL PBS. Then, the microtubes were preincubated at 30°C for 10 min, and PMA was added to the final concentration of 50 μM per sample. After that, the incubation was further prolonged for 5 or 15 min in the dark prior to photoactivation for 15 min using a PMA-Lite^{TM} LED Photolysis Device (Biotium, E90002) at 30°C. Next, the samples were centrifuged at 16,000 × *g* for 10 min at room temperature and subjected to DNA extraction.

DNA Extraction

Extraction of DNA from the PMA-pretreated and untreated samples and from the control *Campylobacter jejuni* subsp. *jejuni* NCTC 11351^T strain was performed as described by Pitcher et al. (1989), with a modification. After the addition of isopropanol, the tubes were placed in the freezer (−20°C) overnight. DNA purity and concentration were determined by spectrophotometry (GE Healthcare Life Sciences, NanoVue, Uppsala, Sweden) (Sambrook and Russell, 2001).

Real-Time PCR

Each assay was conducted in a volume of 25 μL containing 1 × PCR Master Mix (Promega, M750, Madison, WI), 4.5 mM $MgCl_2$, 0.5 μM of each primer, 0.1 μM probe, 0.1 μM ROX reference dye (Life Technologies, 12,223–012, Carlsbad, CA), and an average of 197.25 ng of DNA extracted from each sample. Real-time PCR was performed on an ABI Prism 7500 (Life Technologies). The 287 bp 16S rRNA target of *Campylobacter* spp. was detected using the forward (5′-CTGCTTAACACAAGTTGAGTAGG-3′) and reverse primers (5′-TTCCTTAGGTACCGTCAGAA-3′), and a dark-quenched hydrolysis probe (6FAM-TGTCATCCTCCACGCGGCGTTGCTGC-BBQ) (all from Integrated DNA Technologies, Coralville, IA) (Lubeck et al., 2003; Josefsen et al., 2010; Kruger et al., 2014). Amplification cycles were performed with initial heating at 95°C for 3 min, followed by 45 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. In all the assays, DNA extracted from *C. jejuni* NCTC 11351^T was used as positive control. The data collection was performed in the exponential phase of the reaction. Each sample, PMA-pretreated and untreated, was analyzed in triplicate. Samples with amplification of at least 2 of the 3 replicates were considered positives. Melting point analysis was done after the last cycle to verify the amplification specificity.

To evaluate the limit of detection of the real-time PCR an analytical-sensitivity curve was performed. DNA from *C. jejuni* NCTC 11351^T was diluted in ultrapure water from 20 ng to 2 fg. Given the size of the *C. jejuni* chromosomal DNA of 1,700 kb, it was estimated

Table 1. Detection of *Campylobacter* spp. in frozen and chilled broiler carcasses by real-time PCR associated or not with propidium monoazide (PMA).

Treatment	Non-PMA		PMA treated		n
	Positive	Negative	Positive	Negative	
Frozen	27 (90.00%) [a,x]	3 (10.00%)	2 (6.67%) [b,y]	28 (93.33%)	60
Chilled	26 (86.67%) [a,x]	4 (13.33%)	9 (30.00%) [b,x]	21 (70.00%)	60
Total	53 (88.33%)	7 (11.67%)	11 (18.33%)	49 (81.67%)	120

[a,b]Means followed by different letters in the same row are significantly different ($P < 0.001$; χ^2 test);
[x,y]Means followed by different letters in the same column are different ($P < 0.05$; χ^2 test).

that 20 ng of *C. jejuni* DNA contains approximately 10^7 chromosomal copies (Keramas et al., 2003).

Statistical Analysis

The experiments were conducted in a completely randomized 2 × 2 factorial design, being 2 types of broiler carcasses (frozen and chilled) and 2 pretreatments (treated or not treated with PMA), with 30 samples for each treatment (30 frozen and 30 chilled). Comparisons of detection of *Campylobacter* spp. between frozen and chilled broiler carcasses and between PMA-pretreated and untreated samples were performed by the χ^2 test. Comparisons of quantification of *Campylobacter* spp. between frozen and chilled broiler carcasses and between PMA-pretreated and untreated samples were performed using Tukey's test. In all cases, a significance level of 0.05 was adopted.

RESULTS

The detection limit of the real-time PCR was estimated to be 10 *Campylobacter* genome equivalents. Quantification of the samples was possible for amplification curves with threshold cycle ($\mathbf{C_t}$) values equal to or lower than 37.51. Samples with C_t values higher than 37.51 were classified as negative for *Campylobacter* spp. The enumeration by real-time PCR associated to PMA was always lower than the one made by real-time PCR without PMA pretreatment.

Table 1 summarizes the results of the detection of *Campylobacter* spp. in chilled and frozen broiler carcasses by real-time PCR with or without PMA. Of the 30 frozen samples untreated with PMA, 27 (90.00%) tested positive for *Campylobacter* spp., whereas among the frozen samples submitted to PMA pretreatment, only 2 (6.67%) were positive. Likewise, chilled samples untreated with PMA exhibited a high rate of positivity (86.67%), while chilled chicken samples pretreated with PMA showed only 30.00% of positivity (Table 1).

Among the untreated PMA samples, it was not observed a significant difference in the number of *Campylobacter*-positive broilers comparing frozen and chilled carcasses. On the other hand, among PMA-pretreated samples, a significant higher number of *Campylobacter*-positive samples were observed in the chilled carcasses compared to the frozen samples (Table 1). Comparing only the frozen samples, a significant difference was observed in relation to the PMA-pretreated and untreated ($P < 0.001$). Similarly, the comparison between chilled samples PMA-pretreated and untreated also showed statistical difference ($P < 0.001$; Table 1).

Table 2. Quantification of *Campylobacter* spp. in frozen and chilled broiler carcasses by real-time PCR associated or not with propidium monoazide (PMA).

Treatment	Non-PMA (mean ± SD)	PMA-pretreated (mean ± SD)
Frozen	34.62 ± 1.76 [a,x]	30.58 ± 5.86 [a,y]
Chilled	34.73 ± 1.93 [a,x]	34.89 ± 2.17 [a,x]

[a]Means in the same row were similar according to Tukey's test ($P < 0.05$.
[x,y]Means followed by different letters in the same column are significantly different ($P < 0.05$; Tukey's test).
SD = standard deviation.

The results for the quantification of *Campylobacter* spp. in frozen and chilled chicken carcasses pretreated or not treated with PMA are shown in Table 2. Of the 64 *Campylobacter*-positive samples, 54 had C_t below 37.51 and accordingly conditions to have the contamination load of the microorganism quantified. There was no statistically significant difference in C_t between samples pretreated or not treated with PMA among frozen carcasses, as well as observed for chilled samples. Comparison between frozen and chilled samples not treated with PMA did not yield a statistically significant difference either. Nonetheless, among PMA-pretreated samples, there was a significant difference between the C_t observed for frozen and chilled chicken carcasses (Table 2). The bacterial contamination observed in *Campylobacter*-positive broiler carcasses was between 10^1 and 10^4.

DISCUSSION

Detection and quantification of *Campylobacter* spp. in broilers is important for assessing the level of contamination of chicken meat that reaches the market, especially contamination by viable strains, which are those that pose a real health risk. In the present study, evaluation of the viability of *Campylobacter* spp. in frozen and chilled broiler carcasses by real-time PCR

associated to PMA, it was observed that a large proportion of the chicken samples, especially chilled ones, were contaminated with viable strains of *Campylobacter* spp., thereby representing a potential threat to consumers. Hence, the results highlight the potential public health risk related to the consumption of chicken and the high probability of cross-contamination of other foods by chicken meat.

Moreover, the present finding also showed the usefulness of the real-time PCR associated to PMA for detection and quantification of viable *Campylobacter* spp. in broiler samples. This method can be an great alternative to isolation of the agent that is cumbersome and exhibit low sensitivity due to several characteristics of the pathogen, such as high nutrient requirement, high growth temperature (41°C to 43°C; as opposed to low temperatures used for food preservation), oxygen sensitivity, low competitiveness in relation to other microorganisms and low amount of cells usually present in contaminated food. In addition, it is important to note that the VBNC form that *Campylobacter* spp. can acquire under stress conditions, although retain the infecting potential is not detected by culture and isolation (Barros-Velazquez et al., 1999). In fact, a great correlation between the reference culture-based method and the enumeration of *Campylobacter* spp. by quantitative real-time PCR associated with PMA has been detected (Duarte et al., 2015). Furthermore, several studies have shown that the combination of real-time PCR and pretreatment with PMA or others DNA intercalators are suitable methods to detect viable *Campylobacter* spp. in different types of biological samples (Seinige et al., 2014a; Seinige et al., 2014b; Seliwiorstow et al., 2015). Despite it has not been tested, it is likely that the combination of real-time PCR and PMA also showed superior results to those observed for immunoenzymatic assays, which have showed low sensitivity in the detection of *Campylobacter* spp. in frozen and chilled chicken carcasses compared to other molecular-based techniques (Reis et al., 2018). Thus, the use of DNA intercalators as PMA associated with real-time PCR seems to be a promising method for the detection and enumeration of live *Campylobacter* spp., with effective infectious potential, in chicken meat. Exclusion of broiler batches with high levels of contamination by *Campylobacter* spp. from poultry meat might decrease the risk of human campylobacteriosis.

Freezing has been used in some countries as a strategy to reduce the number of *Campylobacter* spp. in broiler carcasses, since this treatment leads to the decrease of the microbial load by inducing lethal and sublethal injuries to bacterial cells (Frazier et al., 1993; Georgsson et al., 2006; Cox et al., 2014). Indeed, this approach seems to be a suitable procedure to minimize the risks to human health, considering that the present results showed a significantly higher number of samples with viable *Campylobacter* spp. among chilled carcasses compared to frozen chicken (Table 1). These findings are widely corroborated by studies showing that *Campylobacter* spp. are usually more sensitive to freezing than to refrigeration (El-Shibiny et al., 2009; Sampers et al., 2010). However, among PMA-pretreated samples, albeit the chilled carcasses exhibited more *Campylobacter*-positive samples compared to frozen ones. The mean number of bacteria observed per sample was significantly higher in the group of frozen samples. These results may have occurred due to the presence of high numbers of cells with compromised membrane, which could exceed the binding capacity of PMA, allowing DNA amplification of dead or non-viable cells (Luo et al., 2010). Nonetheless, it seems more likely that having only 2 positive samples in the group of frozen chicken carcasses treated with PMA further influenced this result, since one of these samples had a bacterial count equivalent to 10^4, much higher than the range exhibited by the chilled samples (10^1 to 10^2). This hypothesis is evidenced by the high standard deviation of the C_t observed for the frozen broiler samples treated with PMA (Table 2). It is worth noting that very low quantities of *C. jejuni*, approximately 8.0 $\times$ 10^2 CFU mL^{-1}, are sufficient to cause infection and diarrhea in humans (Black et al., 1988).

Regarding the samples not treated with PMA, the results showed that there is no significant difference between frozen and chilled carcasses for microbial DNA detection, which was represented by viable, non-viable and VBNC microorganisms. It can be explained taking into account that the samples used in this experiment were from the same batches and subjected to the same conditions of slaughter.

Comparisons between PMA-pretreated and untreated samples, for both frozen and chilled carcasses, revealed a significantly higher number of *Campylobacter*-positive samples among those submitted only to real-time PCR without PMA pretreatment. These results are easily explained considering that the sum of live and dead microorganisms will always be greater than or equal to the number of live bacteria in paired samples, as the ones used in this study. Nevertheless, despite the fact that the number of *Campylobacter*-positive samples has been significantly higher in broiler carcasses pretreated with PMA compared to the untreated ones, no difference was observed in the amount of bacteria found for both frozen and chilled samples (Table 2). As previously noted, this was probably due to the small number of *Campylobacter*-positive samples among those treated with PMA, especially among the frozen samples. It is expected that if the number of positive samples were higher, there would be a statistical difference between the amount of bacteria detected, considering the pretreated with PMA, which permits quantification of only live cells.

It is important to emphasize that the large number of broiler carcasses contaminated with viable *Campylobacter* spp. and the great quantity of bacteria observed in the positive samples showed by this study, reveal the high level of contamination of the carcasses that occurs during the slaughter of poultry, since after

the cold treatment to preserve the food, *Campylobacter* spp. is no longer able to grow. At temperatures below 30°C, these microorganisms do not multiply (Lee et al., 1998), although maintaining physiological activities important for survival at temperatures of 4°C (Hazeleger et al., 1998).

Overall, the results of the present study indicate that *Campylobacter* spp. remains viable more frequently and in greater amount in chilled broiler carcasses compared to frozen carcasses, showing great hazard to public health. Moreover, the level of *Campylobacter* spp.-contaminated chilled or frozen broiler carcasses was high in the tested sampling.

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