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ABSTRACT

In 2006, the International Organization for Standardization (ISO) published two standard protocols for the detection and enumeration of *Campylobacter* spp. in foods: the ISO 10272-2(direct method) and the ISO 10272-1 (enrichment method). The aim of this study was to compare the efficiency of these two methods in the detection of *Campylobacter* spp. and propose a modification in the enrichment method. Compared with the enrichment method, the direct method yielded a higher number of positive results for *Campylobacter* spp. and, consequently, presented higher sensitivity percentage. The isolation of *Campylobacter* spp. was more difficult when 10 mL of rinse was used in the enrichment method, which is currently recommended by the ISO protocol. Therefore, different rinse volumes were tested (2.5 and 5 mL). The most efficient recovery of *Campylobacter* spp. occurred when 2.5 mL of rinse were used in the enrichment method, most likely due to a lower number of microbial contaminants than that present in the 5 or 10 mL rinses. The proposed modification of the enrichment method will contribute to the food analysis by improving the detection of *Campylobacter* spp. in chicken carcass.

INTRODUCTION

Campylobacter spp. is a common cause of bacterial foodborne illnesses that have been associated with autoimmune diseases, including Guillain-Barre syndrome (GBS), Miller Fisher syndrome, arthritis, and Reiter's syndrome (Oliveira *et al.*, 2005; Snelling *et al.,* 2005). Poultry and poultry products are considered the main vehicle of transmission to humans (Jacobs-Reitsma *et al.,* 2008; Casaril 2010).

The control of *Campylobacter jejuni* contamination in poultry carcasses has increased with the demand of international trade (Scarcelli *et al.,* 2005; Humphrey *et al.,* 2007). Brazil is the largest exporter and Parana state the largest producer of poultry meat in the country (IBGE 2010). The prevalence of *Campylobacter* contamination in poultry carcasses in Brazil ranges from 38 to 93.3% (Maziero & Oliveira 2010; Franchin *et al.,* 2007; Aquino *et al.,* 2002; Dias *et al.,* 1990).

False-negative results have been obtained during the detection of *Campylobacter* spp. in poultry meat due to the nutritional and environmental requirements of these bacteria and to their difficulty in outcompeting bacteria present in the product (Edson *et al.,* 2009). Therefore, the use of adequate enrichment broths, selective media, and conditions that allow the consistent growth of *Campylobacter* spp. are important for their isolation from poultry meat (Habib *et al.,* 2011). In 2006, the International Organization for Standardization (ISO) published the ISO 10272 protocols for the detection and enumeration of *Campylobacter* spp.in foods destined for human and animal consumption. Part 1 of the protocol (ISO 10272-1) describes the detection of *Campylobacter* spp. after selective enrichment of poultry

carcass rinse (enrichment method) and Part 2 (ISO 10272-2) describes the detection and colony count of *Campylobacter* spp. before selective enrichment (direct method).

The aim of this study was to compare the ISO 10272-2 (direct method) and ISO 10272-1 (enrichment method) protocols for the detection of *Campylobacter* spp. in poultry carcasses. Modification of the enrichment method was suggested for better isolation of *Campylobacter* spp. in chicken carcass.

MATERIALS AND METHODS

Sample collection

A total of 80 samples of refrigerated whole broiler carcass from 12 brands were analysed, 5 to 8 samples per brand. The samples were collected in their original packaging from different retailers of Curitiba, Paraná, and sent to the laboratory under refrigeration. After the removal of the packing and the giblets, the samples were placed in sterile plastic bags and weighed. One mL of buffered peptone water (0.1%) was added per gram of sample, and the surface of the carcass was hand massaged for approximately two minutes. Aliquots of rinse were used for the detection of *Campylobacter* spp. before (direct method) and after (enrichment method) selective enrichment.

ISO 10272-2 (Direct Method): Detection and Colony Count of *Campylobacter* spp. before Selective Enrichment

Aliquots of the rinse (0.1 mL) prepared as previously described were streaked in duplicate on mCCDA plates (CM 739) (OxoidInc., Ogdensburg, N.Y., U.S.A.), which were supplemented with cefoperazone (16mg) and amphotericin B (5mg) (SR 155E) (OxoidInc., Ogdensburg, N.Y., U.S.A.), and on modified Bolton agar (MBA) plates. The plates were incubated at 41.5 ± 0.5 °C for 48 h under microaerobic conditions generated by the injection of a mixture of gases (5% O_2, 10% $CO_2$and 85% N_2) (Air Liquide, São Paulo, Brazil) into special jars. The MBA plates and the microaerobic system were prepared as described by Franchin *et al.* (2005).

In each plate, five characteristic colonies were observed under the microscope and evaluated for their characteristic morphology. The colonies with the characteristic morphology were sub-cultured on Trypticase Soy Agar (TSA) plates, which were supplemented with 5% defibrinated sheep blood (Newprov, Curitiba, Paraná, Brazil), and incubated at 36 ± 1 °C for 24 and 48 h under microaerophilic conditions. Subsequently, oxidase and catalase tests were carried out and, when the results were positive, a biochemical identification test was performed using the API CAM system (Biomerieux S.A., Lyon, France). *Campylobacter* was molecularly confirmed by real-time PCR, and quantified using the equation Q = n x 2.5, where n corresponded to the total number of biochemically identified colonies. The results were expressed as CFU/g.

ISO 10272-1 (Enrichment Method): Detection of *Campylobacter* spp. after Selective Enrichment

In 80 whole broiler carcass samples,10-mL aliquots of rinse were transferred to 90 mL of Bolton broth, which was supplemented with cefoperazone (10mg), vancomyc in (10mg), trimethoprim (10mg), and amphotericin B (5mg) (SR 208E) (Oxoid Inc., Ogdensburg, N.Y., U.S.A.). The enrichment broths were incubated at 37 °C for 4 h followed by an additional incubation at 41.5 ± 0.5 °C for 48 h under microaerobic conditions. The enrichment broths were plated onto mCCDA agar and MBA agar plates that were incubated at 41.5 ± 0.5 °C for 48 h under microaerobic conditions. The biochemical identification of *Campylobacter* spp. colonies was performed as described in the ISO 10272-2 protocol (2006). The *Campylobacter* genus was molecularly confirmed by real-time PCR.

ISO 10272-1 (Enrichment Method): Evaluation of different volumes of rinse for detection of *Campylobacter* spp. after Selective Enrichment

In 18 whole broiler carcass samples, the detection of *Campylobacter* spp. by the enrichment method was carried out using different volumes of rinse (2.5, 5.0, and 10.0 mL) added to 90 mL of Bolton broth, as previously described. The selective enrichment, the biochemical identification and the molecular confirmation of *Campylobacte r*were performed as described in section 2.3.

Molecular confirmation of *Campylobacter* spp. by real-time PCR

The molecular confirmation of the colonies plated onto mCCDA agar and MBA agar plates and biochemically identified as *Campylobacter* spp. was performed using TaqMan® real-time PCR.Bacterial DNA extraction was performed using the NewGene Prep and NewGene Preampfrom Simbios Technology (Canoas, Rio Grande do Sul, Brazil).The PCR mixture consisted of a final volume of approximately 30 µL:28µL of TaqMan Master Mix Kit (Simbios Technology) composed of buffer, dNTPs, ultrapure water, primers

and probe, 0.32 µL of Taq polymerase (Simbios Technology),and 2 µL of DNA.

The PCR reactions were carried out in a Thermocycler 7500 (Applied Biosystems, Foster City, CA, U.S.A.) with the following conditions: an initial denaturation step at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, and a final annealing and elongation step at 60 °C for 1 min.

Purified DNA from *Campylobacter jejuni* ATCC 33291 was used as a positive control. Ultrapure water and buffered Master Mix Kit with Taq polymerase were used as negative controls.

Analyses of the results

The prevalence of *Campylobacter* spp. in the chicken meat samples was expressed as a percentage. The sensitivity and specificity of the two methods were calculated according to the model described by Hanrahan & Madupu (1994). The sensitivity and specificity percentages were calculated considering as samples positive for *Campylobacter* those which culture results were confirmed by real-time PCR. Accuracy and correlation analyses were carried out using an Excel spreadsheet (Microsoft), according to models described by Jekel *et al.* (1999).

RESULTS

The comparative results of the direct method (ISO 10272-2) and the enrichment method (ISO 10272-1) as confirmed by real-time PCR of the 80 chicken carcass samples analyzedare shown in Table 1.Twenty samples were positive only by the direct method, five were positive only by the enrichment method, and eight samples were positive by both methods. The sensitivity of the direct and enrichment methods was 84.8% and 39.4%, respectively. The specificity was 100% for both methods.

Table 1 – Comparative analysis of the direct and enrichment methods for the detection of *Campylobacter* spp. in 80 chicken meat samples as confirmed by real-time PCR.

	Direct Method	Enrichment Method
Number of positive samples obtained exclusively by one method	20	5
Number of positive samples obtained by both methods	8	
Sensitivity (%)[1]	84.8	39.4
Specificity (%)[2]	100	100
Prevalence (%)[3]	41.2	

[1] Sensitivity = (true positive results / true positive results+ false negative results) x 100

[2] Specificity = (true negative results / true negative results + false positive results) x 100

[3] Prevalence = (true positive samples x 100) / total number of samples

The overall correlation between the direct and enrichment methods was 64.5%. Therefore, there was little agreement of the results between the ISO 10272-1 and ISO 10272-2 methods. The prevalence of *Campylobacter* spp. was 41.2% (n= 33). *C. jejuni,C. lari, C. upsaliensis* and *C. coli* were identified in 33.7% (n= 27), 3.75% (n= 3), 2.5% (n= 2), and 1.2% (n= 1) of the samples, respectively, .

The results obtained from the 18 chicken meat samples, which enrichment was carried out using different rinse volumes, are shown in Table 2. Six (33.3%) samples were tested positive by the direct method. The numbers of positive samples with rinse volumes of 2.5, 5, and 10 mL were five (27.8%), four (22.2%) and one (5.5%), respectively. Two samples were positive only by the direct method and one sample was positive by the enrichment method with 2.5 mL of rinse. The sensitivity of the direct method was 85.7%, and that of the enrichment method with rinse volumes of 2.5, 5 and 10 mL was 71.4%, 57.1%, and 14.3%, respectively. The specificities of the direct method and the enrichment method with different rinse volumes were 100%.

Table 2 – Comparative analyses of the direct and enrichment methods using different rinse volumes (2.5, 5, and 10 mL) for the detection of *Campylobacter* spp. in18 chicken meat samples as confirmed by real-time PCR.

	Direct Method	Enrichment Method Rinse volumes		
		2.5 mL	5 mL	10 mL
Number of positive samples	6 (33.3%)	5 (27.8%)	4 (22.2%)	1 (5.5%)
Number of positive samples obtained exclusively by one method	2	1	0	0
Sensitivity (%)[1]	85.7	71.4	57.1	14.3
Specificity (%)[2]	100	100	100	100
Prevalence (%)[3]	38.9 (n=7)			

[1] Sensitivity = (true positive results / true positive results+ false negative results) x 100

[2] Specificity = (true negative results / true negative results + false positive results) x 100

[3] Prevalence = (true positive samples x 100) / total number of samples

The results of the seven samples tested positive to *Campylobacter* out of the18 samples analyzed with different volumes of rinse (2.5, 5 and 10 mL) are shown in Table 3. *Campylobacter* was isolated by the direct method in six out of seven positive samples. The best recovery of *Campylobacter* after enrichment was observed with 2.5 mL of rinse. The correlation percentage between the direct method and the enrichment method with 2.5 mL of rinse was 83.3%. In sample 13 (brand E), *Campylobacter* spp. was isolated only after enrichment, possibly due to

the small number of viable cells present in the sample. In the brand D samples, *Campylobacter* spp. was not isolated after enrichment regardless of the rinse volume used possibly due to the high microbial contamination of the chicken carcass samples.

Table 3 – Results of the seven samples that tested positive to *Campylobacter* from 18 samples analyzed using different rinse volumes (2.5, 5 and 10 mL).

			Enrichment Method Rinse volumes		
Samples	Brands	Direct Method	2.5 mL	5 mL	10 mL
7	C	+	+	-	-
9	C	+	+	+	-
10	D	+	-	-	-
12	D	+	-	-	-
13	E	-	+	+	-
14	E	+	+	+	+
15	E	+	+	+	-

DISCUSSION

Compared with the enrichment method, the direct method yielded a higher number of positive results for *Campylobacter* spp. and presented higher sensitivity percentage. Similar results were obtained by Kiess *et al*. (2010), who obtained higher isolation of *Campylobacter* spp. in broiler chicken litter using the direct method. On the other hand, Kuana *et al.* (2008) found no statistically significant difference ($p > 0.05$) between the direct and enrichment methods in cloacae swabs and poultry carcasses.

The results obtained in the present study with the direct method suggest that refrigeration does not affect the recovery of *Campylobacter* spp. Maziero & Oliveira (2010) found no significant difference in the prevalence of *C. jejuni* in chicken meat whether fresh, chilled or frozen, indicating that this bacterium is able to survive low temperature storage conditions.

Although the direct method was more efficient than the enrichment method, it was difficult to count colonies on the mCCDA and MBA plates. Hunt *et al.* (2001) reported that selective media with high water content, such as those used for the isolation of *Campylobacter* spp., can result in colonies that tend to coalesce, making them very difficult to count. Problems with colony counting by the direct method were also observed by Habib *et al*. (2008), after the incubation of 0.3 and 0.4 mL of chicken meat rinse, and the difficulty in counting *Campylobacter* spp. colonies was related to the sample contamination with aerobic mesophilic bacteria (10^5 CFU/g) and *Escherichia coli* (10^3 CFU/g). In the same study, the difficulty in colony counting was observed even when 0.1 mL of chicken meat rinse was used.

Despite the efficiency of the direct method, the exclusive use of this method might compromise the accuracy of the results. The enrichment method detected five samples that were contaminated with *Campylobacter* spp. However, the direct method did not detect the presence of *Campylobacter* spp. in those samples, possibly because contamination was low and it required enrichment (Table 3). Gharst *et al*. (2006), Nauta *et al*. (2009), and Habib *et al.* (2011) obtained higher detection of *Campylobacter* spp. by the enrichment method and concluded that the number of *Campylobacter* spp. colonies in certain samples was not sufficient for the detection by the direct method and that the enrichment of the samples contributed to an increase in the percentage of isolation.

Jacobs-Reitsma *et al* . (2007), who reviewed the ISO 10272 protocol, found that the enrichment method was unsuitable for the isolation of *Campylobacter* spp. from refrigerated chicken meat, and suggested that a culture medium other than mCCDA should be used. In the present study, *Campylobacter* was not isolated in samples tested positive by the direct method after enrichment, regardless of the rinse volume used (Table 3). This have happened possibly due to the inefficacy of the antibiotics present in the enrichment broth (Jacobs-Reitsma *et al.,* 2007) and the inability of *Campylobacter* spp. to outcompete the present microbial contamination (Lee & Newell 2006).

Kiess *et al.* (2010) found no increase in the percentage of *Campylobacter* spp. after enrichment in *Campylobacter Enrichment Broth* (CEB) and suggested that a better recovery could have been obtained if the Preston broth, previously used by Bolton and Robertson (1982), had been used. However, an even better recovery could have been obtained by modifying the proportion between rinse and enrichment broth, e.g., 1:10had been already used by Kuana *et al.* (2008) and Nauta *et al.* (2009), instead of 1:4, used by Kiess *et al.* (2010).

The highest number of positive samples obtained in the present study with the enrichment method using 2.5 mL of rinse suggests that the rinse volume described in the ISO 10272-2 protocol should be modified. In spite of the similar number of positive samples obtained with the direct and the enrichment method using the 2.5 mL rinse, the use of only one method is not recommended because the correlation percentage of 83.3% is not ideal.

It is acknowledged that only a limited number of samples (18 samples) was analyzed in the present study to test the different volumes of rinse for the selective enrichment. The results, however, showed that the volume of 10 mL rinse used in ISO 10272-1 (method Enrichment) could limit the detection of *Campylobacter* spp. and that the use of a smaller volumes of rinse (2.5 mL) could provide a better recovery of *Campylobacter* after selective enrichment.

CONCLUSION

The direct method (ISO 10272-2) was more efficient than the enrichment method (ISO 10272-1) for the detection of *Campylobacter* spp. The use of the 10-mL rinse in the enrichment method, as proposed in the ISO 10272-1 protocol, was less efficient for the recovery of *Campylobacter* spp. than the use of the 2.5-mL rinse. The use of a smaller volume of rinse may be a better option due to the inefficacy of antibiotics to inhibit microbial contaminants, as well as the low competitiveness of *Campylobacter* spp., especially when present in low counts. The correlation percentage of 83.3% between the direct method and the enrichment method with 2.5 mL of rinse indicated that the exclusive use of the direct method may compromise the accuracy of the results.

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