



Detection of *Campylobacter* spp. in chilled and frozen broiler carcasses comparing immunoassay, PCR and real time PCR methods

Luciana Pimenta Reis¹ Liliâne Denize Miranda Menezes² Graciela Kunrath Lima¹
Ethiene Luiza de Souza Santos¹ Elaine Maria Seles Dorneles³ Débora Cristina Sampaio de Assis¹
Andrey Pereira Lage¹ Silvana de Vasconcelos Cançado¹ Tadeu Chaves de Figueiredo^{1*}

¹Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), 31270-901, Belo Horizonte, MG, Brasil. E-mail: tadeu@vet.ufmg.br.

*Corresponding author.

²Instituto Mineiro de Agropecuária (IMA), Contagem, MG, Brasil.

³Departamento de Medicina Veterinária, Universidade Federal de Lavras (UFLA), Lavras, MG, Brasil.

ABSTRACT: In order to detect and identify *Campylobacter* spp. in broiler chicken carcasses, and to compare detection methods, 43 chilled and 43 frozen carcasses were collected and analyzed. Three methodologies were evaluated: an automated Enzyme Linked Fluorescent Assay (ELFA) VIDAS[®]30, Polymerase Chain Reaction (PCR) and real-time PCR. Only four chilled carcasses (4.6%) were considered positive for *Campylobacter* spp. by VIDAS[®]30 and no sample was positive when the conventional PCR technique was used. However, real-time PCR showed a higher incidence of contamination by *Campylobacter* spp. in broiler carcasses, with 45 (52.3%) positive samples. *C. jejuni* was the species most frequently reported in the samples (88.8%). No differences in the frequencies of *Campylobacter* spp. were observed between the chilled and frozen broiler carcasses. In conclusion, real-time PCR was the most sensitive method for the detection of *Campylobacter* spp. in chilled or frozen broiler carcasses, which were mainly contaminated by *C. jejuni*.

Key words: *C. jejuni*, *C. coli*, detection methods, VIDAS[®]30.

Detecção de *Campylobacter* spp. em carcaças de frango de corte resfriadas e congeladas pelos métodos imunoenzimático, PCR e PCR em tempo real

RESUMO: Com o objetivo de detectar e identificar *Campylobacter* spp. em carcaças de frango de corte utilizando três metodologias distintas - ensaio imunoenzimático VIDAS[®]30, Reação em Cadeia da Polimerase (PCR) e PCR em tempo real - foram coletadas e analisadas 43 carcaças de frango resfriadas e 43 congeladas. Quatro carcaças refrigeradas (4,6%) foram consideradas positivas para *Campylobacter* spp. pelo VIDAS[®]30 e nenhuma amostra positiva foi identificada quando utilizada a técnica de PCR. Porém, ao analisar as carcaças pela metodologia da PCR em tempo real, foi observada uma maior incidência de *Campylobacter* spp., com 45 amostras (52,3%) positivas, sendo que *Campylobacter jejuni* foi a espécie mais frequentemente encontrada nas amostras (88,8%). Não foi observada diferença na frequência do micro-organismo entre carcaças de frangos resfriadas e congeladas. Concluiu-se que a técnica de PCR em tempo real apresentou maior sensibilidade na detecção de *Campylobacter* spp. em carcaças de frangos de corte e que foi encontrada elevada presença de carcaças contaminadas, especialmente por *C. jejuni*.

Palavras-chave: *C. jejuni*, *C. coli*, métodos de detecção, VIDAS[®]30.

INTRODUCTION

Campylobacteriosis is a foodborne disease associated with human infection by thermophilic *Campylobacter* spp., mainly *C. jejuni* and *C. coli* (BUTZLER, 2004). These bacteria are some of the most widespread causative agents of human gastroenteritis, with many animals, wild and domestic, serving as potential reservoirs (CDC, 2014). Birds, especially broiler chickens, are the main natural reservoir of *Campylobacter* spp., and there by consumption and handling of poultry and poultry products have been implicated as the main source of infection to humans (HUE et al., 2010, EFSA, 2011, Oliveira et al., 2013). The species that are pathogenic towards humans have

a narrow temperature range for growth (30-46°C) and cannot survive the heating processes used for food preparation (Humphrey et al., 2007).

The detection of *Campylobacter* spp. in food samples is generally performed using traditional culture-based methods, immunoenzymatic assays, or Polymerase Chain Reaction (PCR)-based methods. The traditional culture-based methods for the isolation, identification, and differentiation of *Campylobacter* species are time-consuming and challenging because of the fastidious nature of the microorganisms, which have complex nutritional and environmental requirements (BARROS-VELAZQUEZ et al., 1999; FITZGERALD et al., 2008). The sandwich Enzyme-Linked Immunosorbent Assay (ELISA), which is based on the

detection of an antigenic reaction against the bacteria after their cultivation on selective media, is often used for the analysis of *Campylobacter* spp. in food. This assay is faster, more sensitive, and more specific than the traditional culture-based methods. The Enzyme Linked Fluorescent Assay (ELFA) employs a similar principle featuring a final detection step by fluorescence and is used in the VIDAS® automated immunoassay system (bioMérieux, Marcy-l'Étoile, France) (AOAC, 2015). Methods based on PCR and real-time PCR have also been used for the detection of *Campylobacter* spp. in food samples due to their high specificity and sensitivity. In addition to their great accuracy, PCR-based methods are also faster and more easily performed than the other techniques for detecting *Campylobacter* spp. (SCHNIDER et al., 2010).

Among the control measures that have been recommended to prevent the growth of *Campylobacter* spp. in broiler carcasses, cold treatment is one of the most important. Several studies have reported a significant reduction in the percentage of carcasses contaminated by *C. jejuni* after freezing (EL-SHIBINY et al., 2009; SAMPERS et al., 2010). However, the effects of cooling and freezing on the survival of *Campylobacter* spp. in broiler carcasses have not been completely elucidated.

Therefore, the aims of the present study were to detect and identify *Campylobacter* spp. in chilled and frozen broiler carcasses, and to compare the performance of three detection methods, namely the VIDAS®30 immunoassay method, PCR, and real-time PCR.

MATERIAL AND METHODS

Samples and experimental conditions

Eighty-six broiler carcasses from 43 different production batches were collected in a slaughterhouse industry under the Federal Inspection Service located in Minas Gerais State, Brazil. From each of the 43 batches, one frozen ($n = 43$) and one chilled ($n = 43$) broiler carcass were collected and analyzed on the second day after processing. Hence, each carcass sample represented one batch or one repetition.

Microbiological analyses

VIDAS®30 immunoenzymatic assay

The detection of *Campylobacter* spp. in the broiler carcasses was performed using three distinct methodologies, namely an automated ELFA (VIDAS®30) (AOAC, 2015), PCR (HARMON et al., 1997; VILARDO et al., 2006), and real-time PCR (SCHNIDER et al., 2010).

Each carcass was aseptically placed in a sterile bag containing 100mL of Bolton broth with a selective supplement (SR183E, Oxoid, Basingstoke, UK) and was subjected to a washing process by rinse friction for approximately 1min (bioMérieux, 2015). After rinsing, the solution was incubated at $37 \pm 0.5^\circ\text{C}$ for 4h and then at $42 \pm 0.5^\circ\text{C}$ for 24h under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) [Combibag with microaerophilia generator GENbox (bioMérieux, Hazelwood, USA)]. After incubation, 2mL aliquots of the Bolton broth were transferred to sterile tubes to perform the immunoassay by VIDAS®30 (bioMérieux) and 1.5mL aliquots were transferred to centrifuge microtubes for PCR assays.

The immunoassay was performed with the VIDAS®30 system, using the *Campylobacter* (CAM) kit (bioMérieux), according to the manufacturer's instructions. From the aliquot after incubation in Bolton broth, 0.5mL was heated at 100°C for 15min and then transferred to a test well of a reagent strip and inserted into the VIDAS®30 system. For the confirmation of positive results, a loop from the remaining Bolton broth, that was not heated, was plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid) and incubated at $42 \pm 0.5^\circ\text{C}$ for 48h under microaerophilic conditions (bioMérieux, 2015). Subsequently, colonies with growth typical of *Campylobacter* spp. were selected and plated on blood agar and incubated at $42 \pm 0.5^\circ\text{C}$ for 24h. Then, they were transferred to a *Neisseria-Haemophilus* (NH) card for biochemical tests using the VITEK 2® system (bioMérieux) (VALENZA et al., 2007).

PCR analyses

For *Campylobacter* spp. determination by PCR, the microtubes containing 1.5mL of inoculated Bolton broth were centrifuged at $20,000 \times g$ at room temperature and submitted to DNA extraction (PITCHER et al., 1989). DNA purity and concentration were determined by spectrophotometry (GeneQuant™ 100 Classic, GE Healthcare Life Sciences, Little Chalfont, UK) (SAMBROOK & RUSSELL, 2001).

In the multiplex PCR assays, the primers pg3 (5'- GAACTTGAACCGATTTG-3') and pg50 (5'-ATGGGATTTTCGTATTAAC-3') were used for the amplification of the *flaA* gene (460 bp), which is present in *Campylobacter* species, especially *C. jejuni* and *C. coli*. Additionally, the primers C1 (5'-CAAATAAAGTTAGAGGTAGAATGT-3') and C4 (5'-GGATAAGCACTAGCTAGCTGAT-3') were used for the amplification of a non-determined gene

of *C. jejuni* (160 bp), according to HARMON et al. (1997) and VILARDO et al. (2006) (Table 1). The reactions were performed in a total volume of 25µL containing 20ng of DNA, 1× buffer (10mM Tris-HCl, 50mM KCl) (Phoneutria, Belo Horizonte, MG, Brazil), 5.5mM of MgCl₂ (Phoneutria), 200µM of each deoxyribonucleotide (dATP, dCTP, dGTP, and dTTP), 1.0µM of each primer (Eurofins Scientific, Luxembourg), and 1.25 units of Taq DNA polymerase (Phoneutria). The thermocycling conditions (Veriti® Thermal Cycler, Thermo Fisher Scientific, Waltham, MA, USA) were as follows: initial denaturation step at 94°C for 4min followed by 25 cycles of denaturation at 94°C for 1min, primer annealing at 45°C for 1min, and elongation at 72°C for 1min. The final extension step was performed at 72°C for 7min. The amplified products were submitted to electrophoresis in a 1.5% agarose gel in Tris-borate-EDTA (TBE) buffer (1.0M Tris, 0.01M boric acid, 0.01M EDTA, pH 8.2) and stained with 0.5mg mL⁻¹ ethidium bromide. Products were visualized under ultraviolet light and photographed (L-Pix EX, Locus Biotecnologia, Cotia, SP, Brazil). A 100-bp DNA size marker (Life Technologies, Carlsbad, CA, USA) was used to estimate amplicons length.

Real-time PCR analysis

Real-time PCR assays were performed using the primers Ccj FusA-L1 (5'-GCCTTGAAGAGATTAAAACAGGGATT-3') and Ccj FusA-R1 (5'-TTTAAACGCTGTACCGCAAAGCA-3') to amplify the *fusA* gene of *C. jejuni* (83 bp). In addition, the primers Ccc FusA-L2

(5'-GCCTTGAGGAAATTA AAAACTGGTATT-3') and Ccc FusA-L2 (5'-TTTAAATGCAGTTCCACAAAGCA-3') were used to amplify the *fusA* gene of *C. coli* (83 bp). The probes used were Cj_fusA-probea (FAM-AAGTCTTTCTATCGTTCC-MGBNFQ) for *C. jejuni* and Cc_fusA-probea (NED-AAGTCTTTCTATTGTTCC-MGBNFQ) for *C. coli* (SCHNIDER et al., 2010) (Table 1). Real-time PCR assays were performed with the two primer pairs and probes simultaneously, in triplicate. Reactions were performed in a total volume of 25µL containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.3µM of each primer, 0.2µM of each probe, and 2µL of genomic DNA. Amplification was carried out at 50°C for 2min and 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min using anABI 7500 Thermal Cycler Real-Time PCR System (Applied Biosystems). The result was considered positive when at least two of the triplicate samples were defined as positive, with a threshold cycle (Ct) value less than 40.

Limits of detection of the PCR and real-time PCR assays

To assess the limits of detection of the PCR and real-time PCR assays, two standard curves were generated. One standard curve was performed with serial dilutions of DNA from *C. jejuni* and *C. coli* in ultrapure water. To obtain the other standard curve, DNA from *C. jejuni* and *C. coli* were diluted in Bolton broth that had been used to rinse a broiler carcass. The DNA of *C. jejuni* and *C. coli* were subjected to serial

Table 1 - Primers, probes and sequences for *C. jejuni* and *C. coli* PCR and real-time PCR assays.

Method	Target	Species amplified	Product size (bp)	Primers and probes	Nucleotide sequence (5' to 3')
Multiplex PCR assays	<i>flaA</i> gene	<i>C. coli</i>	460	pg3	GAA-CTT-GAA-CCG-ATT-TG
		<i>C. jejuni</i>	480	pg50	ATG-GGA-TTT-CGT-ATT-AAC
	ND	<i>C. jejuni</i>	160	C-1	CAA-ATA-AAG-TTA-GAG-GTA-GAA-TGT
			160	C-4	GGA-TAA-GCA-CTA-GCT-AGC-TGA-T
Real-time PCR assays	<i>fusA</i> gene	<i>C. jejuni</i>	83	Ccj_fusA-L1	GCCTTGAGGAAATTA AAAACTGGTATT
				Ccj_fusA-R1	TTTAAATGCAGTTCCACAAAGCA
				Cj_fusA-probe	FAM-AAGTCTTTCTATCGTTCC-MGBNFQ
	<i>fusA</i> gene	<i>C. coli</i>	83	Ccc FusA-L2	GCCTTGAGGAAATTA AAAACTGGTATT
				Ccj_fusA-R2	TTTAAACGCTGTACCGCAAAGCA
			Cc_fusA-probea	NED-AAGTCTTTCTATTGTTCC-MGBNFQ	

ND = not determined.

tenfold dilutions from 20ng to 2fg, corresponding to 10^7 to 10^0 genome copies of *Campylobacter* spp., respectively (GenBank accession numbers: *C. jejuni*, CP000814 and *C. coli*, CP006702) (DOLEZEL et al., 2003). Reference strains, which were used as controls in the PCR and real-time PCR assays, were *C. jejuni* (NCTC 11351) and *C. coli* (NCTC 11366). These reference strains were cultivated in blood agar at $42 \pm 0.5^\circ\text{C}$ for 48h under microaerophilic conditions.

Statistical analysis

The experiments were conducted using a completely randomized 2×3 factorial design, including two types of broiler carcasses (chilled and frozen) and three analytical methodologies (immunoenzymatic VIDAS[®]30, PCR, and real-time PCR) with 43 repetitions each. Comparisons among the analytical methodologies were performed by the McNemar test and the analyses of chilled and frozen samples were performed by the Fisher test at the 5% significance level. Statistical analysis was performed using R Statistical Software version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS AND DISCUSSION

Limits of detection of the PCR and real-time PCR assays

In the present study was observed that real-time PCR proved to be more sensitive than conventional PCR to detect *Campylobacter* spp. in broiler carcasses (Table 2). Conventional PCR results using the primers designed by HARMON et al. (1997) and VILARDO et al. (2006) showed that those primer pairs were unsuitable for use with food samples. They were designed for the detection of *C. jejuni* and *C. coli* from cultured bacteria using a large amount of microorganisms per sample (HARMON et al. 1997, VILARDO et al., 2006). Several substances

present in food and clinical materials, such as broiler carcasses, can inhibit or significantly decrease the efficiency of the PCR reactions (SCHRADER et al., 2012). Moreover, it is important to consider that besides the presence of inhibitors, the amount of *Campylobacter* spp. usually present in contaminated food is relatively low, owing to their high sensitivity to atmospheric oxygen concentrations and the low temperatures typically used for food preservation (BARROS-VELAZQUEZ et al., 1999).

Detection of *Campylobacter* spp. using VIDAS[®]30, PCR, and real-time PCR assays

The detection of *Campylobacter* spp. in chicken skin is important for assessing the incidence of contamination among the broiler carcasses that reach the market. Table 3 summarizes results of *Campylobacter* spp. detection in chilled and frozen broiler carcasses. Among the total chilled carcasses, only four (9.3%) samples were considered positive for *Campylobacter* spp. by the VIDAS[®]30 assay and no sample was considered positive when the conventional PCR technique was used. However, results of the real-time PCR showed a higher incidence of *Campylobacter* spp. in broiler carcasses, with 45 (52.3%) samples being considered positive, being 24 (55.8%) from chilled and 21 (48.8%) from frozen carcasses. Hence, the results of the present study highlighted the potential public health risks linked to the consumption of chicken and the usefulness of the real-time PCR assay for the detection of *Campylobacter* spp. in broiler carcasses.

Detection of *C. jejuni* and *C. coli* by real-time PCR

The real-time PCR assay allowed differentiation between *C. jejuni* and *C. coli* and showed that *C. jejuni* was the most frequently detected *Campylobacter* species reported in the tested samples (Table 4). *C. jejuni* was detected in

Table 2 – Limit of detection of the PCR and real-time PCR assays used to detect *Campylobacter* spp. in broiler carcasses.

Primers	Dilution	Analytical sensitivity	Number of genome copies	Technique	Specificity
pg3 / pg50	Water	20 pg	10^4	PCR	<i>Campylobacter</i> spp.
pg3 / pg50	Broiler carcass rinse ^a	200 pg	10^5	PCR	<i>Campylobacter</i> spp.
C1 / C4	Water	200 pg	10^5	PCR	<i>C. jejuni</i>
C1 / C4	Broiler carcass rinse	2 ng	10^6	PCR	<i>C. jejuni</i>
FusA-L1 / FusA-R1	Water	200 fg	10^2	Real-time PCR	<i>C. jejuni</i>
FusA-L1 / FusA-R1	Broiler carcass rinse	200 fg	10^2	Real-time PCR	<i>C. jejuni</i>
FusA-L2 / FusA-R2	Water	200 fg	10^2	Real-time PCR	<i>C. coli</i>
FusA-L2 / FusA-R2	Broiler carcass rinse	200 fg	10^2	Real-time PCR	<i>C. coli</i>

^aBroiler carcass rinse performed with Bolton broth.

Table 3 – Detection of *Campylobacter* spp. in chilled and frozen broiler carcasses using the immunoassay method VIDAS[®]30, PCR and real-time PCR.

Treatment	n	-----VIDAS-----				-----PCR-----				-----Real-time PCR-----			
		Pos		Neg		Pos		Neg		Pos		Neg	
		n	%	n	%	n	%	n	%	n	%	n	%
Chilled	43	4	9.3	39	90.7	0	0	43	100	24	55.8	19	44.2
Frozen	43	0	0	43	100	0	0	43	100	21	48.8	22	51.2
Total	86	4	4.6	82	95.4	0	0	86	100	45	52.3	41	47.7

McNemar $\chi^2 = 2.25$; $P = 0.1336$; $gl = 1$; VIDAS[®]30 x PCR.

McNemar $\chi^2 = 39.0244$; $P = 4.185 \times 10^{-10}$; $gl = 1$; VIDAS[®]30 x real-time PCR.

McNemar $\chi^2 = 43.0222$; $P = 5.412 \times 10^{-11}$; $gl = 1$; PCR x real-time PCR.

Fisher test: VIDAS – chilled x frozen – $P = 0.1162$; real-time PCR – chilled x frozen $P = 0.0625$.

Pos = positive for *Campylobacter* spp.

Neg = negative for *Campylobacter* spp.

40 (88.8%) of the 45 positive samples, while *C. coli* was found in 15 (33.3%) samples. *C. jejuni* and *C. coli* were simultaneously reported in ten (22.2%) of the 45 positive broiler carcasses. Among the chilled carcasses that were positive for *Campylobacter* spp., *C. jejuni* was detected in 20 carcasses (83.3%) and *C. coli* was detected in 9 carcasses (37.5%). *C. jejuni* and *C. coli* were simultaneously detected in five chilled carcasses (20.8%). Among the frozen carcasses that were positive for *Campylobacter* spp., *C. jejuni* was detected in 20 carcasses (95.2%) and *C. coli* was reported in six carcasses (28.5%). Both *C. jejuni* and *C. coli* were simultaneously detected in five frozen samples (23.8%). It is noteworthy that, usually, 95% of human cases of campylobacteriosis are related to infection by *C. jejuni*, while *C. coli* accounts for 4% of cases and *C. lari* accounts for about 1% (NACHAMKIN et al., 2008).

Comparison among the methods used for *Campylobacter* spp. detection

A comparison among the three methods used for *Campylobacter* spp. detection in broiler carcasses showed a significant difference ($P < 0.001$) between real-time PCR and the other studied techniques (Table 3). The VIDAS[®]30 immunoenzymatic assay did not present superior results to those of conventional PCR for the detection of *Campylobacter* spp. in broiler carcasses. The low bacterial counts in the carcasses could have influenced the performance of the VIDAS[®]30 assay in this study, since only four chilled carcasses were found to be positive for contamination when using this technique. These results may suggest that the VIDAS[®]30 immunoenzymatic assay was only able to detect contamination in carcasses with higher loads

of microorganisms. Thus, comparing results of the VIDAS[®]30 assay with those of real-time PCR, the lower incidence of *Campylobacter* spp. detected by the former method may suggest that the bacterial counts in the tested broiler carcasses were below the limit of detection ($> 10^3$ CFU mL⁻¹) (SAHIN et al., 2008) or that viable but non culturable bacteria predominated in the samples. Low incidences of the contamination of broiler carcasses by *Campylobacter* spp. recorded by PCR and VIDAS[®]30 immunoenzymatic assay was unexpected, since high rates of contamination has been widely demonstrated in the world and in different Brazilian states (DIAS et al., 1990; FREITAS & NORONHA, 2007; KAAKOUSH et al., 2015).

Findings from real-time PCR showed a completely different situation, i.e., that a high incidence (52.3%) of broiler carcasses were contaminated by *Campylobacter* spp.. These results most probably reflect the high analytical sensitivity of the method, which has a limit of detection of 10^2 cells mL⁻¹. Lower detection limit of real-time PCR as compared to those of conventional PCR (10^4 – 10^6 cells mL⁻¹) and the VIDAS[®]30 assay ($> 10^3$ CFU mL⁻¹) (SAHIN et al., 2003) presumably enabled the detection of *Campylobacter* spp. in samples in which the concentration of microorganisms was relatively low, which is the typical situation in food samples. It should be noted that amplification by real-time PCR was observed even for low concentrations of *Campylobacter* spp. DNA (20fg, equivalent to 10 cells mL⁻¹). Moreover, the Bolton broth showed no inhibitory effects during the assessment of the detection limit of the real-time PCR assays. However, since the efficiency of the reaction with this lowest tested concentration of DNA was substantially lower

Table 4 - Detection of *C. jejuni* and *C. coli* in chilled and frozen broiler carcasses.

Treatment	----- <i>C. jejuni</i> / <i>C. Coli</i> -----		----- <i>C. jejuni</i> -----		----- <i>C. coli</i> -----		Total n
	n	%	n	%	n	%	
Chilled	5	20.8	20	83.3	9	37.5	24
Frozen	5	23.8	20	95.2	6	28.5	21
Total	10	22.2	40	88.8	15	33.3	45

than that observed for the other tested concentrations, these results were disregarded when interpreting the limit of detection. It is also important to consider that very low quantities of *C. jejuni*, around 8.0×10^2 CFU mL⁻¹, are sufficient to cause infection and diarrhea in humans (BLACK et al., 1988).

Regarding the temperature treatment of broiler carcass, it is interesting to observe that despite the higher frequency of carcasses contaminated by *Campylobacter* spp. among chilled samples compared to frozen ones (Table 3), this difference was not significant ($P > 0.05$). The fact that real-time PCR detects the genetic material of the microorganism, even if it is no longer viable, could be the reason for the absence of a difference between these storage conditions, since *Campylobacter* spp. is usually more sensitive to freezing than refrigeration (EL-SHIBINY et al., 2009; SAMPERS et al., 2010). It is possible to speculate that if the immunoenzymatic technique used had shown a higher analytical sensitivity, or if we had incorporated conventional methods for the isolation of *Campylobacter* spp., a significantly higher proportion of positive samples among chilled carcasses would have been observed. Therefore, although the real-time PCR assay detected a high level of *Campylobacter* spp. contamination among broiler carcasses, it is not possible to make statements about the viability of this microorganism. Thus, it is not possible to assess the real risk to public health using real-time PCR alone. Alternative methods for the detection of viable *Campylobacter* spp. cells have been researched. Combination of real-time PCR with propidium monoazide was tested by Josefsen et al. (2010) and provided a fast and reliable methodology for detection and quantification of viable *Campylobacter* bacteria in broiler carcasses.

CONCLUSION

Real-time PCR was more sensitive than conventional PCR and the VIDAS®30 immunoassay for the detection of *Campylobacter* spp. in broiler carcasses. The level of *Campylobacter* spp.

contamination among chilled or frozen broiler carcasses was high, especially for *C. jejuni*.

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