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ORIGINAL ARTICLE

Quantification of *Campylobacter* spp. in chicken carcass rinse by real-time PCR

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Abstract

Aims: In this study, a real-time quantitative polymerase chain reaction (PCR) method was examined for its ability to quantify *Campylobacter* spp. in chicken carcass rinses and compared with bacteriological culturing.
Methods and Results: The linearity of the real-time PCR quantification protocol was assessed on pure DNA. The amplification efficiency was 100% and the square regression coefficient (R^2) was 0·998. Quantification was linear over at least 7 log units. Using a crude cell lysate gave the highest sensitivity and the detection limit of the method was 3·3 log CFU per carcass. The statistical correlation between the bacteriological enumeration and the real-time quantitative (Q)-PCR determined using chicken carcasses sampled at the end of the slaughter line was 0·733. The difference in detection levels was probably because of the detection of stressed, dead or viable but not culturable cells by Q-PCR.
Conclusion: The real-time Q-PCR method described in this study is a powerful tool for determining the number of *Campylobacter* cells on carcasses.
Significance and Impact of the Study: The real-time Q-PCR method is available to quantify the *Campylobacter* contamination at the slaughterhouse level and could be used to evaluate primary production.

Introduction

Campylobacter spp. and in particular the thermotolerant species, *Campylobacter jejuni* and *Campylobacter coli*, are recognized worldwide as a leading bacterial cause of diarrhoea and food-borne gastro-enteritis (Dekeyser *et al.* 1972; Allos and Blaser 1995; Solomon and Hoover 1999). The high incidence of clinical disease associated with this organism, its low infective dose in humans and the possibility of making patients more susceptible to develop postinfectious diseases like Guillain Barré syndrome, makes *Campylobacter* infections one of the major public health issue (Tauxe 1992). Foods of animal origin, in particular poultry, have been identified as a significant risk factor for human infection (Nadeau *et al.* 2003; Nielsen *et al.* 2006) as a result of infection and contamination at the farm and slaughterhouse levels (Herman *et al.* 2003; Rasschaert *et al.* 2006; VanWorth *et al.* 2006).

The prevalence of *Campylobacter* in broiler flocks and on broiler carcasses is very high and was estimated by a German study to be respectively 41·1% and 45·9% (Atanassova and Ring 1999). In a Belgian study in 18 individual broiler flocks, 7 flocks were positive for *Campylobacter* at the end of rearing and the carcasses of 13 flocks were found positive after slaughter (Herman *et al.* 2003). *Campylobacter* primarily colonizes the large intestine, caeca and cloaca of broilers where 10^5–10^9 CFU g^{-1} of faecal content have been observed (Corry and Atabay 2001). At the slaughterhouse level, around 20% of the carcasses were contaminated and about 20·8% of the filets at retail are contaminated in Belgium (Anonymous 2006). The number of *Campylobacter* found in retail chicken products may exceed 10^3 CFU per 100 g meat or 10^2–10^5 CFU per carcass (Stern and Pretanik 2006). Seasonal differences in the numbers of *Campylobacter* have been reported with a higher number in the summer (Hutchison *et al.* 2006).

For the implementation of effective control measures in the primary production and as a tool in quantitative microbial risk assessment studies it is necessary to have a sensitive detection method and in particular a quantitative result. *Campylobacter* detection and enumeration in foods by traditional culture methods is problematic owing to the fragility of this organism (Solomon and Hoover 1999). DNA-based methods like polymerase chain reaction (PCR) have been increasingly used for rapid, sensitive and specific detection of *Campylobacter*. For the quantification of *Campylobacter* in faeces (Lund *et al.* 2004; Rudi *et al.* 2004) or in chicken rinses (Yang *et al.* 2003; Wolffs *et al.* 2005), real-time PCR methods are already described. The technology of real-time PCR has tremendous potential for detection and identification. For the quantification aspects, further research is required before implementation of the technology. Different steps have to be optimized such as: (i) the quantitative lysis of the bacteria in the matrix, (ii) concentration of the sample to obtain a higher detection limit that is important for the quantification of low numbers of cells and (iii) the elimination of PCR inhibitors. This study evaluated different primers already described for the detection of *Campylobacter* spp. or *C. jejuni*. Various DNA isolation protocols were used taking into account the detection limit and inhibition in the PCR reaction. The results of the real-time quantitative PCR assay on chicken carcass rinses were correlated with the results of a traditional culturing method.

Materials and methods

Bacterial isolates

The bacterial isolates used in the specificity study are listed in Table 1. Isolates were stored at −80°C in brain heart infusion broth (BHI; Oxoid, Basingstoke, England) supplemented with 5% defibrinated horse blood (E&O laboratories, Bonnybridge, Scotland) at −80°C using 15% (w/v) glycerol as cryoprotectant. The isolates were recovered from −80°C storage and grown on sheep blood agar plates. *Campylobacter* isolates were incubated under microaerophilic conditions (at 5% O_2, 10% CO_2, 85% N_2 in an O_2/CO_2 incubator; Thermo Forma, Ohio, USA) at 42°C for 42 h. Other bacterial strains were incubated either aerobically or anaerobically, as appropriate, at 37°C. The cells were scraped from the plates and DNA was prepared by using the method described by Flamm *et al.* (1984).The study comprised 28 *C. coli* isolates from different sources (human cases in Belgium, Canada, Brazil and South Africa, animal or environment-related strains), 38 *C. jejuni* isolates from poultry and farm environment, human cases and 16 other *Campylobacter* spp. The specificity of the primers was tested on 18 non-*Campylobacter* DNA templates.

Carcass rinse samples

A whole broiler carcass was purchased in a local supermarket and divided in two parts in the lab. From the two parts of the chicken carcass the bacteriological contamination was determined. One part was used to determine the natural contamination while the other part was spiked with known quantities of *C. jejuni* (MB 3390 and MB 1263).

Three hundred millilitre of phosphate-buffered saline (pH 7·3; BR0014G, Oxoid) was added to one-half of the carcass. The surface of the carcass was hand massaged for 1 min and subsequently the carcass was shaken gently at 150 rev min^{-1} for 1·5 min in the buffer. For bacteriological enumeration of *Campylobacter*, 10 ml of the carcass rinse was transferred to 90 ml of *Campylobacter*-selective Preston broth [(nutrient broth no. 2, Preston *Campylobacter*-selective supplement (Oxoid), 5% lysed horse blood (E&O Laboratories, Bonnybridge, Scotland)] and incubated for 24 and 48 h at 42°C under microaerophilic conditions (as before). This method allows a semi-quantitative estimation with a limit of detection of 30 CFU for a half carcass (1 CFU per 10 ml × 300 ml). After selective enrichment, a loop of 10 μl was streaked on modified charcoal cefoperazone desoxycholate agar (mCCDA, CM739; Oxoid) with addition of the CCDA-selective supplement (SR155; Oxoid). Also 1 ml and 100 μl of the carcass rinse were directly spread on mCCDA and 100 μl of a serial dilution was spread on mCCDA by a spiral plater (Eddy Jet IUL Instruments, Heusden-Zolder, Belgium). The detection limits of these methods are respectively 300 and 3000 CFU for a half carcass. After 24 and 48 h of microaerophilic incubation at 42°C of the mCCDA plates, glazy colonies were microscopically investigated for the presence of *Campylobacter*-like cells. For further identification of the isolates on *Campylobacter* genus level, the 16S rDNA-based primer pair C412F (5′-GGATGACACTTTTCGGAGC-3′) and C1288R (5′-CATTGTAGCACGTGTGTC-3′) was used with an annealing temperature of 55°C in the PCR reaction (Linton *et al.* 1996).

DNA isolation of carcass rinse

The carcass rinse was filtered through a Whatman cellulose filter number 4 (Whatman, Kent, UK) to remove fat, blood and skin particles. Subsequently, the filtrate was

Table 1 List of bacterial strains used to test the specificity and the sensitivity of the various real-time polymerase chain reaction (PCR) primers

Strain	Original no.	R-camp F2-R2 Sybr Green	R-camp F2-R2 TaqMan	R-camp F1-R1 Sybr Green	R-Cj-Ox F1-R1
Campylobacter coli	LMG 15882	+	+	+	−
	LMG 15905	+	+	+	−
	LMG 6440^T	+	+	+	−
	LMG 8530	+	+	+	−
	LMG 9220	+	+	+	−
	LMG 9799	+	+	+	−
	R 2152	+	+	+	−
	R 2618	+	+	+	−
	R 12819	+	+	+	+
	R 23929	+	+	+	−
	R 24238	+	+	+	−
	MB 2053	+	+	−	−
	MB 2063	+	+	−	−
	MB 2068	+	+	+	−
	MB 2072	+	+	+	−
	MB 2076	+	+	+	−
	MB 2080	+	+	+	−
	R 3520	+	+	+	−
	R 23927	+	+	+	−
	R 23928	+	+	+	−
	R 24232	+	+	+	−
	R 20934	+	+	+	−
	R 24720	+	+	+	−
	16/F	+	+	+	−
	21/P	+	+	+	−
	K33/1	+	+	+	−
	K40/2	+	+	+	−
	KC 7	+	+	−	−
Campylobacter jejuni subsp. *jejuni*	LMG 8841^T (MB 1263)	+	+	+	+
	LMG 9879	+	+	−	−
	LMG 9880	+	+	+	+
	ATCC 33291	+	+	+	+
	KC1	+	+	+	+
	KC 19	+	+	+	+
	KC 42	+	+	+	+
	KC 43	+	+	+	+
	KC 52	+	+	+	+
	KC 61	+	+	+	+
	KC 63·2	+	+	+	+
	KC 69·1	+	+	−	−
	KC 71	+	+	+	+
	KC 86·2	+	+	+	+
	KC 87·1	+	+	−	−
	KC 126·1	+	+	−	−
	KC 127·1	+	+	−	+
	KC 132	+	+	−	−
	KC 128	+	+	+	+
	31/F	+	+	−	−
	MB 3390	+	+	+	+
	R 16191	+	+	+	+
	R 23957	+	+	+	+
	R 23960	+	+	−	+
	R 23961	+	+	+	+
	R 2631	+	+	+	+

Table 1 *Continued*

Strain	Original no.	R-camp F2-R2 Sybr Green	R-camp F2-R2 TaqMan	R-camp F1-R1 Sybr Green	R-Cj-Ox F1-R1
	R 8259	+	+	+	+
	R 9305	+	+	−	+
	R 9310	+	+	+	+
	LMG 15906	+	+	+	+
	LMG 9081	+	+	−	+
	LMG 14245	+	+	+	+
	LMG 8842	+	+	+	+
	3/481	+	+	+	+
Campylobacter	LMG 8556	+	+	+	+
jejuni subsp. *doyley*	LMG 9130	+	+	+	+
	LMG 8870	+	+	−	+
	R 23954	+	+	+	+
Campylobacter lanienae	R 23962	+	+	−	−
Campylobacter lari	LMG 9253	+	+	+	−
	LMG 11760	+	+	+	−
	LMG 9889	+	+	+	−
	LMG 8845	+	+	−	−
	LMG 8846^{T}	+	+	+	+
	R 11303	+	+	+	−
	R 3017	+	+	+	−
Campylobacter law renceae	LMG 23081	+	+	−	−
Campylobacter upsaliensis	LMG 7915	+	+	−	−
	LMG 13945	+	+	−	−
Campylobacter concisus	LMG 14004	+	−	−	+
Campylobacter hyointestinalis	LMG 10891	+	+	−	−
	KC 94	+	+	−	−
	KC 123	+	+	−	−
Campylobacter insulaenigrae	LMG 22716	+	+	−	−
Arcobacter shirrowii	LMG 6621^{T}	+	−	−	−
Arcobacter cryaerophilus	LMG 9904^{T}	+	−	−	−
Arcobacter butzleri	LMG 10828^{T}				
Helicobacter pullorum	LMG 16318	−	−	−	−
	KC 112	−	−	−	−
Bifidobacterium bifidum	LMG 10645	−	−	−	−
Citrobacter diversus	LMG 5519	−	−	−	−
Enterobacter aerogenes	LMG 2094	−	−	−	−
Escherichia coli	LMG 2092^{T}				
Klebsiella pneumonia	LMG 3080	−	−	−	−
Lactobacillus acidophilus	MB 2783	−	−	−	−
Proteus mirabilis	LMG 3257^{T}	−	−	−	−
Pseudomonas aeruginosa	LMG 6395	−	−	−	−
Salmonella Enteritidis	LMG 10395	−	−	−	−
Salmonella Typhimurium	LMG 10396	−	−	−	−
Serratia liquefaciens	LMG 7884	−	−	−	−
Staphylococcus aureus	LMG 8064	−	−	−	−
Streptococcus thermophilus	LMG 13101	−	−	−	−

Strains were from the BCCM/LMG Bacteria Collection.

Ghent R, the research collection of the BCCM/LMG Bacteria collection; MB, bacterial collection of the institute for Agricultural and Fisheries Research Unit Technology and Food; KC, the research collection of strains isolated during the survey of 18 broiler flocks (Herman *et al.* 2003).

centrifuged for 10 min at 10 000 ***g***, the pellet was dissolved in 2 ml of PBS (Oxoid) and lysis followed by incubation in a water bath of 95°C for 17 min. After lysis, the cell debris was centrifuged by a short spin (8000 rcf for 5 s). This crude cell lysate was used in the real-time quantitative (Q)-PCR (5 and 1 μl) giving a theoretical detection limit of respectively 400 and 2000 CFU per half carcass. The theoretical detection limit was calculated supposing that one DNA copy could be detected in the PCR reaction and that there was no moss of DNA during the extraction. If 1 μl is used in the quantitative (Q)-PCR, the calculation is (1 CFU $\mu l^{-1} \times 2000$ μl) and when 5 μl is used in the Q-PCR (1 CFU/5 $\mu l \times 2000$ μl) the detection limit is five times lower. A phenol/chloroform extraction and a precipitation of the DNA with ethanol followed according to the methods described in Sambrook *et al.* (1989) for concentration and purification of the DNA present in this crude cell lysate. The DNA pellet was dissolved in 50 μl of water and either 5 or 1 μl was used in the real-time Q-PCR and resulting in a theoretical detection limit of respectively 10 and 50 CFU per half carcass as calculated before, 1 CFU/5 μl used in the PCR from 50 μl DNA extract that came from one half of the chicken carcass or 1 CFU $\mu l^{-1} \times 50$ μl.

Calculation of the linearity of the real-time PCR on pure culture and chicken rinses

For the validation of the real-time PCR quantification method a linear relation is required between the *Ct* value and the amount of added DNA copies of a known strain (strains MB 1263 and MB 3390). First, DNA was prepared from *c.* 1×10^9 CFU ml^{-1} of *C. jejuni* by the method of Flamm *et al.* (1984). The concentration of the purified DNA was determined by measuring the optical density at 260 nm with a spectrophotometer (Merck, Overijse, Belgium). The number of genomic DNA copies was calculated by the equation $M = n \times (1.013 \times 10^{-21} \mathrm{gbp}^{-1})$ with n as the total number of base pairs (bp), which is for *Campylobacter* determined to be 1777 831 bp (Fouts *et al.* 2005). Isolated DNA was serially diluted ten-fold in water and subjected to real-time Q-PCR.

The carcass rinse DNA prepared from a negative chicken carcass sample was spiked with DNA of MB 1263 and *C. jejuni* subsp. *jejuni* MB 3390. A tenfold serial dilution series of the carcass DNA was made starting from $1{\cdot}5 \times 10^7$ DNA copies in the PCR to 0. A standard curve was generated, which is the linear correlation between the number of added DNA copies and the corresponding *Ct* value that was obtained by real-time Q-PCR.

Real-time Q-PCR

Different published primer pairs (Table 2) were first tested for specificity and sensitivity using all strains listed in Table 1. First, SYBR Green I was used as detection system. The real-time PCR assay was carried out in a 25-μl volume and contained $1 \times$ SYBR Green I master mix (Applied Biosystems, Foster City, USA), primers (final concentration of 600 nmol l^{-1} of each primer; Eurogentec, Seraing, Belgium) and 5 μl of template DNA (standardized photometrically at 5 ng μl^{-1}). Besides these published primers, PCR primers were designed with Primer Express software (Applied Biosystems) based on a putative oxidoreductase subunit of *C. jejuni* (GenBank accession number AL111168; Nayak *et al.* 2005).

The primers and probe described by Lund *et al.* (2004) were also used in the Taqman assay. This real-time Q-PCR assay was also carried out in a 25-μl volume and contained $1 \times$ Taqman master mix (Applied Biosystems), primers and Taqman probe described by Lund *et al.* (2004; final concentration of 600 nmol l^{-1} of each primer and 200 nmol l^{-1} for the probe; Eurogentec), and 5 or 1 μl of template DNA (standardized photometrically at 5 ng μl^{-1}). Real-time quantification was performed on the ABI 7000 sequence detection system using the SDS 1·0 application software (Applied Biosytems) with the following amplification programme: an initial activation step of the enzyme at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 1 min annealing and elongation at 60°C. The amplification efficiency (E) was calculated from the standard curves using the equation: $E = (10^{-1/\mathrm{slope}} - 1) \times 100$.

Naturally contaminated broiler carcasses

To compare the real-time PCR quantification method with the common bacteriological enumeration, 19 broiler

Table 2 Primers and probe used in the real-time polymerase chain reaction (PCR) quantification of *Campylobacter*

Detection	Primer or probe name	Primer or probe sequence (5′ → 3′)	Reference
Campylobacter genus	R-campF2	CAC GTG CTA CAA TGG CAT AT	Lund *et al.* 2004
	R-campR2	GGC TTC ATG CTC TCG AGT T	
	R-campP2	*CAG AGA ACA ATC CGA ACT GGG ACA	
Thermotolerant Campylobacter	R-campF1	CTG CTT AAC ACA AGT TGA GTA GG	Josefsen *et al.* 2004
	R-campR1	TTC CTT AGG TAC CGT CAG AA	
Campylobacter jejuni	R-Cj-Ox-Fw1	AGG GCT TCT AGG TGG TTC TGT AGT AG	This study
	R-Cj-Ox-R1	TGC GTA AGT TCT GCT GCC TTT	

*Carboxyfluorescein was used as reporter dye and on the 3′ end a black whole quencher (BHQ-1) was present.

carcasses were sampled at the slaughterhouse. The *Campylobacter* status of the flock was first determined at the farm by analysing collected caecal droppings. At the slaughterhouse, the carcasses were collected at two different points at the end of the slaughter line after chilling. First, the carcasses were sampled immediately after cooling to 7°C for 1 h 45 min ($n = 9$). Second, carcasses were sampled after they passed a cooling tunnel at −10°C for 20 min with air streaming ($n = 10$). On arrival at the laboratory, the carcasses were immediately analysed by plating and by real-time Q-PCR. The procedure of the crude cell lysate was used and 1 μl was used in the real-time Q-PCR reaction.

Statistical analysis

The *Campylobacter* counts were expressed as the $\log_{10}$ of the CFU per chicken carcass (or half of the carcass during the optimization of the method) both after plating and after real-time Q-PCR. For the latter, an obtained *Ct* value was translated on the basis of the standard curve to the number of DNA copies in 1 μl of crude cell lysate used in the PCR reaction. This number was then multiplied by 2000 to calculate the number of CFU in 300 ml of carcass rinse (derived from half of the carcass) or by 4000 to calculate the number of CFU per carcass. A two-sided *t*-test for independent samples has been performed to compare carcass counts after the first cooling and these after the second cooling by either plating or real-time Q-PCR. To compare carcass counts obtained by real-time Q-PCR and enumeration, a two-sided *t*-test for dependent samples has been performed and standard Pearson correlation coefficient (R) and the associated significance level were calculated. All analyses were carried out using Statistica 7·1 (StatSoft, Tulsa, OK, USA).

Results

Testing of different primer pairs for the detection of *Campylobacter* spp.

The sensitivity and specificity of different primers were first tested using SYBR Green I (Tables 1 and 3). The highest sensitivity was obtained with the primers published by Lund *et al.* (2004) but with this primer pair amplification was also obtained for *Arcobacter* spp. (Tables 1 and 3). Therefore, the Taqman assay with the described primers and probe (R-camp F2, R-camp R2 and R-camp P2) was used resulting in a specific reaction for all *Campylobacter* spp. and no cross-reaction with other closely related *Campylobacter* sp. This real-time Q-PCR assay was further used for the quantification of *Campylobacter* on chicken carcasses.

Determination of the linearity and quantification range of the real-time PCR method

DNA isolated from *c.* 1×10^9 CFU ml^{-1} *C. jejuni* (MB 3390) was 10-fold serially diluted in water and subjected to real-time Q-PCR. The standard curve based on these dilutions of DNA starting from $1{\cdot}5 \times 10^7$ to 1·5 DNA copies showed a linear relationship between log input DNA and the threshold cycle. The regression equation was $y = -3.3026x + 39.216$ with a square regression coefficient of $R^2 = 0{\cdot}998$ and an efficiency of amplification (E) of 100%.

Optimization of the method for quantification of *Campylobacter* on chicken carcasses

A DNA isolation method was optimized for the real-time PCR quantification of *Campylobacter* on chicken carcasses. It was noticed that high amounts of blood, skin particles and fat caused inhibition in the real-time PCR reaction; therefore, samples were shortly centrifuged to remove eukaryotic cellular debris.

Adding serially diluted DNA of MB 3390 to the crude cell lysate of a bacteriological negative carcass rinse resulted in a linear relationship between the added DNA copies and the *Ct* value ($y = -3{\cdot}396x + 39{\cdot}39$ with $R^2 = 0{\cdot}994$) and an amplification efficiency of 96·9%. However, variations were noticed when a ten-fold serial dilution starting from $3{\cdot}75 \times 10^7$ DNA copies to 3·75 DNA copies was added to cell lysates of various carcasses (Fig. 1). In one occasion, a difference of five *Ct* values was measured compared with the control DNA clearly

Table 3 Evaluation of the different primer pairs (sensitivity and specificity) for the detection of *Campylobacter* spp. by using SYBR Green I

Primer pair	*Campylobacter coli*	*Campylobacter jejuni*	*Campylobacter lari*	*Campylobacter lanienae*	Other *Campylobacter* spp.	Non-*Campylobacter* spp.
R-camp F1-R1	25/28	27/38	5/7	0/2	1/7	–
R-camp F2-R2	28/28	38/38	7/7	2/2	7/7	+ *Arcobacter* spp.*
R-Cj-Ox F1-R1	1/28	34/38	1/7	0/2	1/7	–

*By using the Taqman assay, no cross-reaction with other species closely related to *Campylobacter* was observed.

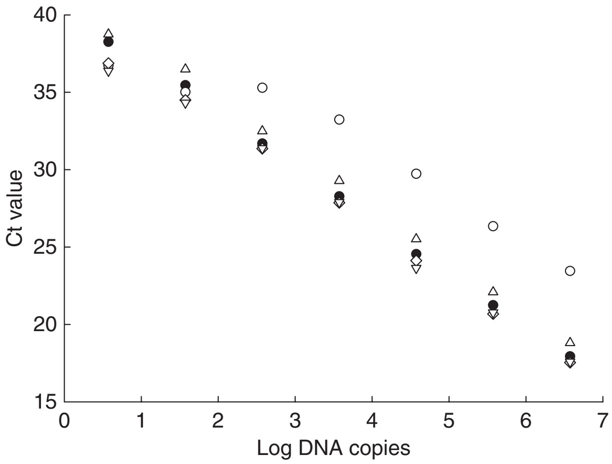

Figure 1 The linear relationship between the log input DNA (starting from 6·57 to 0·57) in pure DNA samples and in the presence of crude cell lysate of four different chicken carcasses. (●), Pure DNA; (○), Carcass 1; (△), Carcass 2; (◇), Carcass 3; (▽), Carcass 4.

Table 4 The log DNA copies detected after using 5 and 1 μl in the real-time polymerase chain reaction. The concentrated carcass DNA was spiked with a known quantity of DNA corresponding to log 6·54 copies

Carcass	5 μl	1 μl
1	4·17	6·28
2	3·35	6·43
3	5·04	6·34
4	3·36	6·41

demonstrating PCR inhibition but which is linear in all the dilutions. Either 1 or 5 μl of purified and concentrated DNA were tested in the PCR. By using 5 μl in the PCR reaction, a clear inhibition of the PCR reaction is noticed that was not observed when testing 1 μl (Table 4).

When adding $7{\cdot}9 \times 10^4$ CFU of a pure *Campylobacter* culture to the carcass rinse (300 ml), theoretically 39·5 DNA copies should be found in 1 μl of the crude cell lysate. However, a corresponding amount of only 3·9 DNA copies were detected by real-time Q-PCR, i.e. a 1 log difference. By using 1 μl of the purified DNA of the same crude cell lysate, theoretically $1{\cdot}6 \times 10^2$ DNA copies should be detected. In this case however, only 4·21 DNA copies were found by real-time PCR. In a second experiment, the carcass rinse (300 ml) was inoculated with $1{\cdot}23 \times 10^6$ CFU. Theoretically, by using 1 μl in the PCR reaction of the crude cell lysate, 615 DNA copies should be detected. This corresponds very well with the 521 DNA copies detected corresponding to $1{\cdot}04 \times 10^6$ CFU. If 1 μl of the purified and concentrated DNA was used in the real-time PCR reaction $3{\cdot}02 \times 10^4$ CFU were detected. It can be concluded that the real-time PCR method starting from crude cell lysates is the most sensitive one. From Fig. 1 it is clear that for three carcasses no inhibition is observed and on average 87% of the added *Campylobacter* cells were detected whereas for carcass number 4 a clear inhibition is observed. During this optimization period one half of the carcasses were tested for natural contamination by *Campylobacter*. On 70% of the carcasses at retail level *Campylobacter* was detected by traditional culturing method including enrichment. The contamination level was low and ranged between 30 and 300 CFU for one half of the carcass (Table 5). By real-time PCR, much higher contamination levels were found ranging between $6{\cdot}06 \times 10^2$ CFU and $2{\cdot}78 \times 10^5$ CFU per carcass half.

Table 5 Quantification of *Campylobacter* (CFU per carcass half) on carcasses bought at a supermarket by bacterial counting (enumeration on mCCDA and enrichment in Preston) and by quantification using real-time quantitative-polymerase chain reaction (PCR) in which 1 μl of the crude cell lysate was used in the PCR reaction

Sample	Bacterial counting	Real-time Q-PCR
1	30 ≥ 300	$1{\cdot}35 \times 10^4$
2	30 ≥ 300	$2{\cdot}70 \times 10^5$
3	30 ≥ 300	$2{\cdot}78 \times 10^5$
14	30 ≥ 300	$6{\cdot}06 \times 10^2$

Comparison of carcass contamination by *Campylobacter* at the end of the slaughter line by real-time Q-PCR and by enumeration on mCCDA

In total 19 carcasses were sampled, 9 carcasses after the first cooling and another 10 carcasses after the second cooling. The mean carcass count by real-time Q-PCR was 7·95 (±0·48) log CFU after the first cooling and 8·52 (±0·72) log CFU after the second cooling. By enumeration, mean counts of 4·44 (±0·72) log CFU were obtained after the first cooling and 4·83 (±0·93) log CFU after the second cooling. Both using real time Q-PCR and enumeration, statistical analysis showed that there is not enough evidence to conclude that mean counts obtained after the first and second cooling were different ($P > 0{\cdot}05$).

Data of carcasses obtained after the first and second cooling were therefore combined and the results obtained by enumeration and real-time Q-PCR were compared (Fig. 2). By real-time Q-PCR, the *Campylobacter* counts detected on the carcasses was on average 8·25 (±0·66) log CFU whereas by enumeration on mCCDA much lower counts of *Campylobacter* were detected averaging 4·64

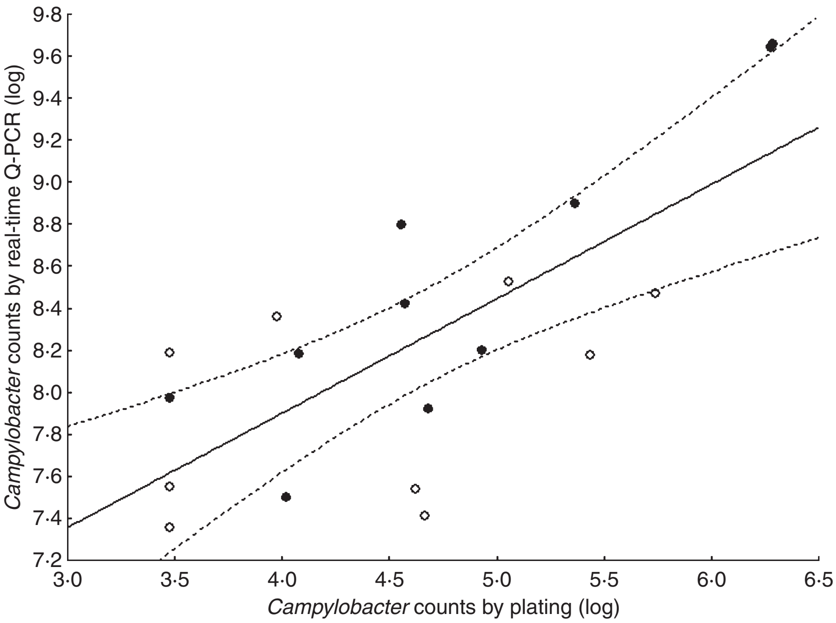

Figure 2 The correlation between the real-time quantitative-polymerase chain reaction and the bacteriological enumeration of 19 chicken carcasses sampled direct after cooling (n = 9; ○) and after the carcasses passed a cooling tunnel with air streaming (n = 10; ●). (- - -), 95% confidence.

(±0·90) log CFU ($P < 0{\cdot}001$). A clear positive association is apparent between the bacteriological counts and the real-time Q-PCR counts ($R = 0{\cdot}733$, $P < 0{\cdot}001$).

Discussion

Several case control studies showed that the most important vehicles for human infections with *Campylobacter* are contaminated poultry products (Rosenquist *et al.* 2003; Wingstrand *et al.* 2006). A reduction in the *Campylobacter* concentration on chicken carcasses could help significantly in reducing the number of human campylobacteriosis cases. A quantitative microbial risk assessment study by our group demonstrated that a 60%, 78% and 97% reduction in human campylobacteriosis cases in Belgium can be achieved by reducing the carcass contamination by 1, 2 and 3 log CFU, respectively (Messens *et al.* 2007; Gellynck *et al.* 2008). To carry out such a study, quantitative data of *Campylobacter* on carcasses are necessary (Uyttendaele *et al.* 2006). Therefore, it would be beneficial to have a rapid, sensitive and specific method to quantify *Campylobacte*r on carcasses. Several real-time PCR methods for the detection of *Campylobacter* are described but were not used for quantification. In our study, different primers that were already described were compared and the most sensitive and specific real-time PCR method was used to validate the quantification of *Campylobacter* on carcasses. The real-time PCR quantification assay (Lund *et al.* 2004) using the Taqman probe was evaluated as the most sensitive and specific one for detection of *Campylobacter*. SYBR Green I could not be used because cross-reaction was observed with other species closely related with *Campylobacter*.

To validate the quantification of *Campylobacter* on carcasses the linear relationship between the log input DNA and the cycle threshold was determined first for pure *Campylobacter* DNA and later on in the presence of carcass DNA. It was clear that using 1 μl of the crude cell lysate in the PCR reaction offers the best results. To evaluate the carcass DNA preparation step and the quantitative lysis of the bacteria both a high and a low amount of *Campylobacter* cells was added to a negative carcass sample. A good linear relationship was obtained for the different samples, which demonstrates the efficiency of the DNA isolation protocol in combination with the real-time PCR quantification. For one carcass a clear inhibition in the real-time Q-PCR was observed that resulted in an underestimation of the real *Campylobacter* contamination level. A further concentration of the carcass DNA shows a clear inhibition of the real-time Q-PCR reaction that results in an underestimation of the real amount of *Campylobacter* cells present on the carcass. These findings emphasize the need of adding an internal amplification control (IAC) to interpret the results obtained by Q-PCR correctly (Hoorfar *et al.* 2004). However, it is important to test whether the addition of the IAC does not compromise the Q-PCR performance. The detection limit obtained by the real-time Q-PCR method was *c.* 2000 CFU per carcass, which corresponds to *c.* 7 CFU ml^{-1} rinses. The sensitivity obtained by our method is in the same range as those in earlier published papers of 10 CFU ml^{-1} (Hong *et al.* 2007) and

20 CFU ml^{-1} (Best *et al.* 2003). In general, lower detection sensitivity was observed when the microbiological method was used in comparison with the real-time quantification method. At the slaughterhouse level, a clear positive association is apparent between the bacteriological enumeration and the real-time Q-PCR counts. Counts obtained by real-time Q-PCR were however 3 log units higher than those obtained by enumeration on mCCDA. This was also observed in other studies where in general 1 log higher counts are detected by real-time Q-PCR in comparison with the conventional enumeration methods (Hong *et al.* 2007). This phenomenon is not only seen for *Campylobacter* but also for the enumeration of *Vibrio parahaemolyticus* in seafood (Takahashi *et al.* 2005). The presence of viable but not culturable or dead *Campylobacter* spp. (Tholozan *et al.* 1999) could be the reason for the underestimation but also the fragility and the difficulties to enumerate *Campylobacter* could be the basis for the observed differences. Direct cooling of the carcasses results in a rapid decrease of the cultivable bacteria but no significant differences were noticed between the first and the second cooling. The carcass contamination determined by real-time Q-PCR was 8·25 log CFU per carcass. The reason of this high contamination level is probably because of the sampling of carcasses from a *Campylobacter*-positive flock status at the slaughterhouse level. In the United States, a contamination level of 3·05 log CFU per carcass is reported (Stern and Robach 2003) but also large variations were noticed between different carcasses from 2·3 to 7·72 CFU per carcass (Stern *et al.* 2007). These values are within the same order as the ones we found by bacteriological enumeration.

From this study, we can conclude that a real-time quantification PCR protocol is available for the enumeration of *Campylobacter* on chicken carcasses. At the slaughterhouse level, a clear positive association is apparent between the bacteriological enumeration and the real-time Q-PCR counts. Counts obtained by real-time Q-PCR were however higher than those obtained by enumeration on mCCDA. Direct enumeration of *Campylobacter* by real-time Q-PCR could be useful to follow the initial carcass contamination at the slaughterhouse level. It is a sensitive, specific and rapid method to investigate, for example whether intervention measures taken at the farm also result in lower initial carcass contamination levels.

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