

## Quantitative Detection of *Campylobacter jejuni* on Fresh Chicken Carcasses by Real-Time PCR

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### ABSTRACT

*Campylobacter jejuni* infection is a significant cause of foodborne gastroenteritis worldwide. Consumption and handling of poultry products is believed to be the primary risk factor for campylobacteriosis. Risk assessments require quantitative data, and *C. jejuni* is enumerated usually by direct plating, which sometimes allows growth of non-*Campylobacter* bacteria. The objective of the present study was to develop a quantitative real-time PCR method (q-PCR) for enumerating *C. jejuni* in chicken rinse without a culturing step. The procedure to obtain the template for the PCR assay involved (i) filtration of 10 ml of chicken rinse, (ii) centrifugation of the sample, and (iii) total DNA extraction from the pellet obtained using a commercial DNA extraction kit. The detection limit of the method was comparable to that for plating 100  $\mu$ l of chicken rinse on modified charcoal cefoperazone deoxycholate agar, and the detection limit could be further improved 10-fold by concentrating the DNA eluate by ethanol precipitation. A close correlation for spiked chicken rinse was obtained for the results of the quantitative real-time PCR method and direct plating ( $r = 0.99$ ). The coefficient of correlation for the methods was 0.87 when samples from chicken carcasses on the slaughter line were analyzed, whereas a lower correlation ( $r = 0.76$ ) was obtained when samples from retail carcasses were analyzed. Greater variation in the proportion of dead and/or viable but not culturable *Campylobacter* types in the retail samples may explain the decreased correlation between the methods. Overall, the new method is simple and fast and the results obtained are closely correlated with those for direct plating for samples containing a low proportion of dead *Campylobacter* cells.

*Campylobacter* is the most frequently reported bacterial agent causing acute infective diarrhea in Sweden and in other countries within the European Union (1). Approximately 90% of Swedish patients with reported campylobacteriosis are infected by *Campylobacter jejuni*, whereas *Campylobacter coli* is found in the vast majority of the remaining 10%. An even higher proportion of *C. jejuni* infection is recorded when *C. jejuni* and *C. coli* are differentiated by PCR instead of the commonly used phenotypic hippurate test, which tends to give false-negative results by misclassifying *C. jejuni* isolates as *C. coli* (13).

Consumption and handling of chicken products have been indicated by numerous case-control studies as common sources of infection (5, 11, 16, 17). The intestinal tract of chickens frequently contains *Campylobacter*, and cross-contamination during transport and processing from chickens with high intestinal bacterial counts is likely to result in contaminated carcasses. In a quantitative risk assessment, Rosenquist et al. (14) found that changes in *Campylobacter* counts on the carcass have a larger impact on the incidence of human campylobacteriosis than do changes in *Campylobacter* prevalence. Thus, methods for enumerating *Campylobacter* on meat and other foods and in drink items are required.

Most traditional culture methods for analyzing *Campylobacter* in foods include an enrichment step, which elim-

inates the possibility of enumeration. Enrichment is used to enhance the detection limit and to prevent growth of non-*Campylobacter* species, which are commonly present, when direct plating is performed. Alternatives to direct plating are methods based on the most-probable-number technique, which are sensitive but labor intensive.

Recently, real-time PCR assays have gained wide interest as a technique for quantitative detection of bacteria in complex matrices, and several methods for enumerating bacteria in food have been described (2, 3, 7, 15, 19). Quantitative real-time PCR (q-PCR) methods for *Campylobacter* also have been described (9, 12, 18, 20). However, to our knowledge, there is no previous report in which q-PCR and direct plating methods used to enumerate *Campylobacter* on naturally contaminated food have been fully compared. Consequently, the objective of the current study was to develop a method whereby a previously described q-PCR assay (12) could be applied for detection and quantification of *C. jejuni* present on spiked and naturally contaminated chicken carcasses and the results of this method could be compared with those of direct plating.

### MATERIALS AND METHODS

***Campylobacter*-spiked chicken rinse.** A fresh chicken carcass was placed in double plastic bags, and 400 ml of buffered peptone water (pH 7.2) was added. The carcass was shaken for 1.5 min, and the rinse fluid obtained was checked for the presence of *Campylobacter* by direct plating and q-PCR. *Campylobacter*-negative chicken rinse was spiked with *C. jejuni* strain C31 from

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the National Food Administration (Uppsala, Sweden). This strain was originally isolated from a chicken carcass and has a pulsed-field gel electrophoresis genotype frequently found in Sweden. Strain C31 stored at  $-70^{\circ}\text{C}$  was streaked on blood agar and grown microaerobically (85%  $\text{N}_2$ , 5%  $\text{O}_2$ , and 10%  $\text{CO}_2$ ) at  $41^{\circ}\text{C}$  overnight and then restreaked and incubated for an additional 16 to 18 h before colonies were harvested with a cotton swab. The bacteria were suspended in a 0.85% NaCl solution, and the bacterial concentration was determined by measuring the optical density (OD) at  $\lambda = 600\text{ nm}$  with an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del.). Results from direct plating indicated that an  $\text{OD}_{600}$  of 0.46 corresponded to  $1.28 \times 10^9$  CFU  $\text{ml}^{-1}$ . For experiments, 100  $\mu\text{l}$  of a 10-fold dilution series ranging from  $2 \times 10^8$  to  $2 \times 10^3$  CFU  $\text{ml}^{-1}$  were transferred and carefully mixed into test tubes containing 20 ml of chicken rinse.

**Naturally contaminated chicken.** Chicken carcasses were purchased from different retail supermarkets in Uppsala. On arrival at the laboratory, the carcasses were stored at  $6^{\circ}\text{C}$  and analyzed on the same day or the following day. The carcasses from slaughterhouses were analyzed within an ongoing study by the National Veterinary Institute (Uppsala, Sweden). The carcasses were taken from the slaughter line after chilling and were rinsed as described for spiked chickens. The chicken rinses were sent in an insulated box with refrigerant gel packs to the National Veterinary Institute, and on the following day samples were directly plated. Unused chicken rinse was stored frozen ( $-20^{\circ}\text{C}$ ), and 30 *Campylobacter*-positive chicken rinse samples were later sent frozen to the National Food Administration and analyzed using the q-PCR method.

**Direct plating.** Direct plating was performed by spreading 0.1 ml of rinse fluid on modified charcoal cefoperazone deoxycholate agar (mCCDA; Oxoid, Basingstoke, UK) with automatic plate-spreading equipment (Eddy Jet, IUL Instruments, Barcelona, Spain). The plates were subsequently left on the bench for 30 min with the lid half open before being incubated microaerobically at  $41^{\circ}\text{C}$ . Counting was performed after 24 and 48 h of incubation.

**DNA isolation.** Ten milliliters of chicken rinse was filtered through a nylon mesh with a pore size of 100  $\mu\text{m}$ , the filtrate was centrifuged ( $5,000 \times g$  for 5 min), and the supernatant was discarded. The pellet was subjected to total DNA extraction using a commercial DNA purification kit (DNeasy tissue kit, Qiagen, Crawley, UK) following the manufacturer's instructions for gram-negative bacteria. For experiments with concentrated DNA eluate, an ethanol precipitation step was performed by adding 400  $\mu\text{l}$  of 95% EtOH and 8  $\mu\text{l}$  of 5 M NaCl to 190  $\mu\text{l}$  of purified DNA. The samples were subsequently vortexed and centrifuged (14,000 rpm  $\text{min}^{-1}$  for 10 min), and the supernatant was discarded. The pellets obtained were air dried and resuspended in 19  $\mu\text{l}$  of distilled water.

**The q-PCR assay.** The optimized PCR mixture (25  $\mu\text{l}$ ) contained a 0.15  $\mu\text{M}$  concentration of the *C. jejuni*-specific probe (5'-TCT CCT TGC TCA TCT TTA GGA TAA ATT CTT TCA CA-3'), which was labeled at the 5' end with the reporter dye carboxyfluorescein and at the 3' end with the quencher 6-carboxy-*N,N,N',N'*-tetramethylrhodamine, 0.5  $\mu\text{M}$  concentrations of the forward primer (5'-CTG AAT TTG ATA CCT TAA GTG CAG C-3') and the reverse primer (5'-AGG CAC GCC TAA ACC TAT AGC T-3'), 12.5  $\mu\text{l}$  of TaqMan Universal PCR master mix (Applied Biosystems, Foster City, Calif.), and 5  $\mu\text{l}$  of template. Probe and primers have been described previously by Nogva et al. (12). The amplification profile was 1 cycle at  $95^{\circ}\text{C}$  for 10 min and 45

cycles at  $95^{\circ}\text{C}$  for 20 s and  $60^{\circ}\text{C}$  for 1 min. A standard curve ranging from 1- to 6-log copies of the *C. jejuni* genome was included in duplicate. The DNA concentration in the standard samples was determined spectrophotometrically, and the number of genome copies was calculated as previously described (10), assuming that one *C. jejuni* genome has a mass of 1.7 fg. The q-PCR experiments were conducted on an ABI 7500 instrument (Applied Biosystems), and the raw data obtained were analyzed with the enclosed 7500-system software, version 1.3.1.

**Calculation of PCR efficiency.** The experiment for efficiency determination was performed, as described for the q-PCR, with DNA templates extracted from *C. jejuni*-spiked chicken rinse and from a pure culture of *C. jejuni*. The cycle threshold ( $C_T$ ) was plotted against the log concentration of added template. The slope of the regression line obtained was used for calculating the efficiency ( $E$ ):  $E = [10^{(-1/\text{slope})}] - 1$ .

## RESULTS

**The q-PCR method.** The main steps in the method developed are (i) shaking the carcass in buffered peptone water to get the bacteria in a liquid phase, (ii) filtration of the liquid obtained through a mesh to remove larger particles of meat and fat, (iii) concentration of all cells present in the sample by centrifugation, (iv) use of a commercial extraction kit to extract the total DNA from the cells present in the pellet, and (v) analysis of the purified DNA by q-PCR.

**Amplification efficiency and repeatability of the q-PCR assay.** The PCR assay described by Nogva et al. (12) was optimized and performed best with a final primer concentration of 0.5  $\mu\text{M}$  and a probe concentration of 0.15  $\mu\text{M}$  (data not shown). Addition of  $\text{MgCl}_2$  above the concentration present in the ready-made universal PCR master mix did not improve the PCR efficiency (data not shown).

The PCR efficiency in the optimized assay was determined by constructing a standard curve. The template DNA was extracted from chicken rinse spiked with *C. jejuni* and serially diluted 10-fold in eluate from DNA extract of *Campylobacter*-negative chicken rinse. The  $C_T$  value was plotted against the 10-fold serial dilution of DNA (Fig. 1). The efficiency of the PCR amplification was calculated at 96% by using the slope of the regression line obtained. DNA extracted from a pure culture of *C. jejuni* and serially diluted 10-fold in pure water was also analyzed, and the slope of the plotted curve also indicated an efficiency of 96% (data not shown). The difference in  $C_T$  values between the two curves ( $\Delta C_T$ ) at each concentration was plotted to determine how close to parallel the two curves were. The regression line obtained had a slope close to zero ( $-0.0065$ , data not shown), indicating that potential PCR inhibitors present in the chicken rinse were effectively removed during the DNA extraction procedure.

The repeatability of the q-PCR assay and direct plating was investigated. Chicken rinse spiked with *C. jejuni* at  $10^3$  CFU  $\text{ml}^{-1}$  was analyzed 10 times with both methods. The coefficient of variation was 14% for the q-PCR method and 5% for direct plating (data not shown). A coefficient of variation of 6% was obtained when DNA from one of the extractions was analyzed 10 times by the q-PCR assay.

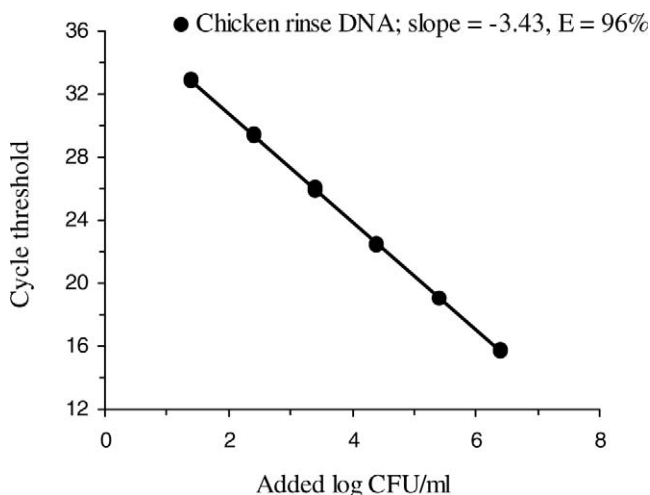


FIGURE 1. The efficiency (*E*) of the PCR assay. *C. jejuni* DNA extracted from chicken rinse and serially diluted 10-fold in DNA from an extraction of *Campylobacter*-free chicken rinse was analyzed with q-PCR. Each concentration of DNA was analyzed in triplicate.

Thus, DNA extraction and the q-PCR assay contributed equally to the overall variation in the q-PCR method.

**Detection limit of the q-PCR method compared with direct plating.** The detection limit was determined using DNA extracted from chicken rinse spiked with three low concentrations of *C. jejuni*. Four of five q-PCR assays detected *C. jejuni* in chicken rinse spiked with 2.6 CFU ml<sup>-1</sup>, whereas no signal was obtained for chicken rinse spiked with 10% of this number of bacteria (Table 1).

Five chicken rinse samples spiked with the same concentration of *C. jejuni* were analyzed with direct plating and q-PCR in parallel. One DNA extraction was performed per sample, and the purified DNA was analyzed in duplicate by q-PCR. For direct plating, 100 μl of chicken rinse was spread on each of four mCCDA plates per sample. Four plates were used because the concentration was close to the detection limit. *C. jejuni* was detected in all 10 PCR assays, and 17 of the 20 plates grew colonies of *Campylobacter* after incubation (Fig. 2A). The *Campylobacter* concentration in the five samples was 10 to 33 CFU ml<sup>-1</sup> by direct plating and 18 to 42 CFU ml<sup>-1</sup> by the q-PCR method (Fig. 2B).

In one experiment, eluate from the DNA extraction was concentrated 10 times by ethanol precipitation to improve

TABLE 1. q-PCR analysis of chicken rinse samples spiked with three different concentrations of *C. jejuni*

Sample no.	<i>C<sub>T</sub></i> values in samples spiked with <i>Campylobacter</i> at:		
	0.2 CFU ml <sup>-1</sup>	2.6 CFU ml <sup>-1</sup>	24 CFU ml <sup>-1</sup>
1	ND <sup>a</sup>	38.5	36.3
2	ND	39.1	35.8
3	ND	ND	36.1
4	ND	37.9	35.9
5	ND	39.8	36.0

<sup>a</sup> ND, not detected.

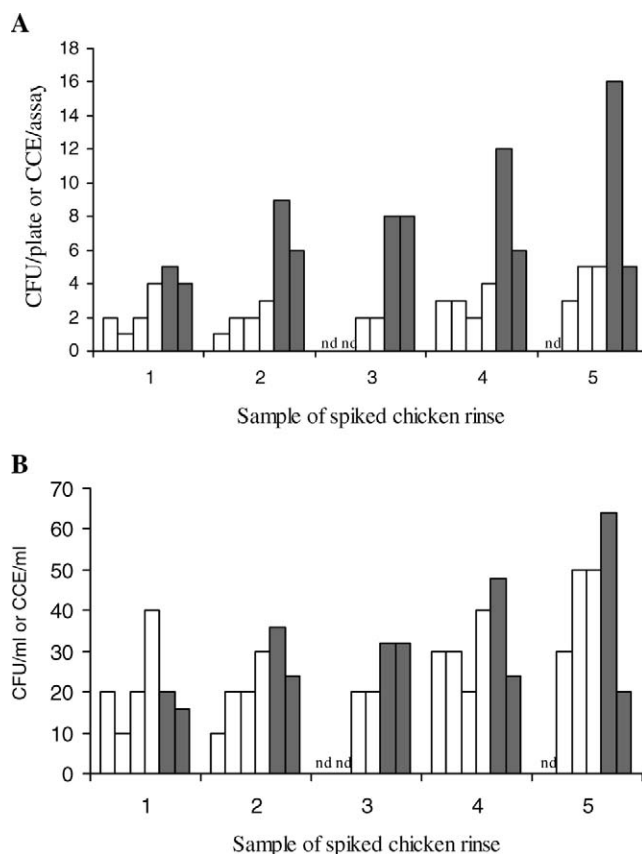


FIGURE 2. Five chicken rinse samples with the same concentration of *C. jejuni* were analyzed by direct plating and q-PCR in parallel. Open bars indicate the number of colonies per plate (A) or per milliliter of chicken rinse (B), and shaded bars indicate the number of *Campylobacter* cell equivalents (CCE) per q-PCR assay (A) or per milliliter of chicken rinse (B). nd, not detectable.

the detection limit. The reduced number of PCR cycles obtained with the concentrated DNA corresponded well with the degree of concentration, and the reduction in *C<sub>T</sub>* values was constant over a 5-log range (Fig. 3). Ethanol precipitation was necessary to detect *C. jejuni* in the chicken rinse spiked with the lowest concentration (Fig. 3).

**Correlation of results for the q-PCR method and direct plating.** Quantification of the bacteria in the chicken rinse samples spiked with 10-fold dilutions of *C. jejuni*

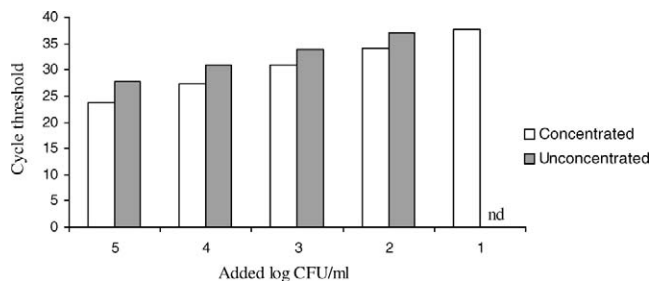


FIGURE 3. Part of the DNA extracted from chicken rinse spiked with concentrations of *C. jejuni* ranging from 1 to 5 log CFU was concentrated 10-fold by ethanol precipitation. The number of PCR cycles needed for detection was compared for nonconcentrated and concentrated DNA. nd, not detectable.

ranging from  $10^1$  to  $10^6$  CFU ml<sup>-1</sup> was performed with the q-PCR method and direct plating. The experiment was repeated five times, and the log CFU per milliliter from direct plating (y axis) were plotted against the log *Campylobacter* cell equivalents (CCE) per milliliter from the q-PCR (x axis) (Fig. 4A). A linear correlation with a correlation coefficient of 0.99 was observed between the methods, and the slope of the regression line was 1.0. The concentration of *Campylobacter* was consistently a 0.5-log value higher with direct plating method than with the q-PCR method (Fig. 4A).

The q-PCR assay was performed on 30 samples of naturally contaminated chicken rinse originating from carcasses at slaughter that were positive for *Campylobacter* by direct plating. For these samples, the coefficient of correlation was 0.87 between the two methods (Fig. 4B).

Thirteen of the 45 samples of mainly whole chicken carcasses from retail outlets tested positive for *Campylobacter* with both direct plating and the q-PCR method. Six samples tested positive only with the q-PCR method, and one sample was positive with direct plating but negative with the q-PCR method. The remaining 25 samples tested negative with both methods. Three positive samples could not be enumerated by direct plating because of partial overgrowth by other species on the mCCDA plates. The quantitative correlation between the two methods for the 10 samples for which quantitative data were obtained is shown in Figure 4C. The slope of the regression line was 1.6, and the correlation coefficient was 0.76.

## DISCUSSION

The PCR assay is generally robust but can be markedly inhibited by substances commonly present in food matrices, leading to a reduced detection limit and an underestimation of the target concentration (4). The high PCR efficiency (96%) obtained for both pure DNA and DNA extracted from the matrices indicates that potential inhibitors present in the chicken rinse were removed during the DNA purification step. The decrease in the  $C_T$  values for the ethanol-precipitated DNA corresponded well to the degree of concentration (10-fold), also indicating efficient removal of potential inhibitors. The relatively short length (86 bp) of the amplified DNA sequence might contribute to the robustness and high efficiency observed.

For direct plating, 100  $\mu$ l of chicken rinse usually is spread onto each plate, resulting in a detection limit of 10 CFU ml<sup>-1</sup>. The q-PCR method developed has a theoretical detection limit of 4 CFU ml<sup>-1</sup> (200  $\mu$ l DNA eluate is obtained from 10 ml of chicken rinse, and 5  $\mu$ l is used as template). The detection limit of the q-PCR method was in the same range or slightly better than that for direct plating. This finding indicates that the majority of the *Campylobacter* cells were lysed, and a high yield was obtained with the DNA purification procedure used. The detection limit can be improved for direct plating by spreading 1 ml on a large mCCDA plate. A similar improvement of the detection limit can be achieved for the q-PCR method by concentrating the 200  $\mu$ l of DNA eluate to 20  $\mu$ l by ethanol precipitation.

The correlation coefficient ( $r = 0.99$ ) for q-PCR and

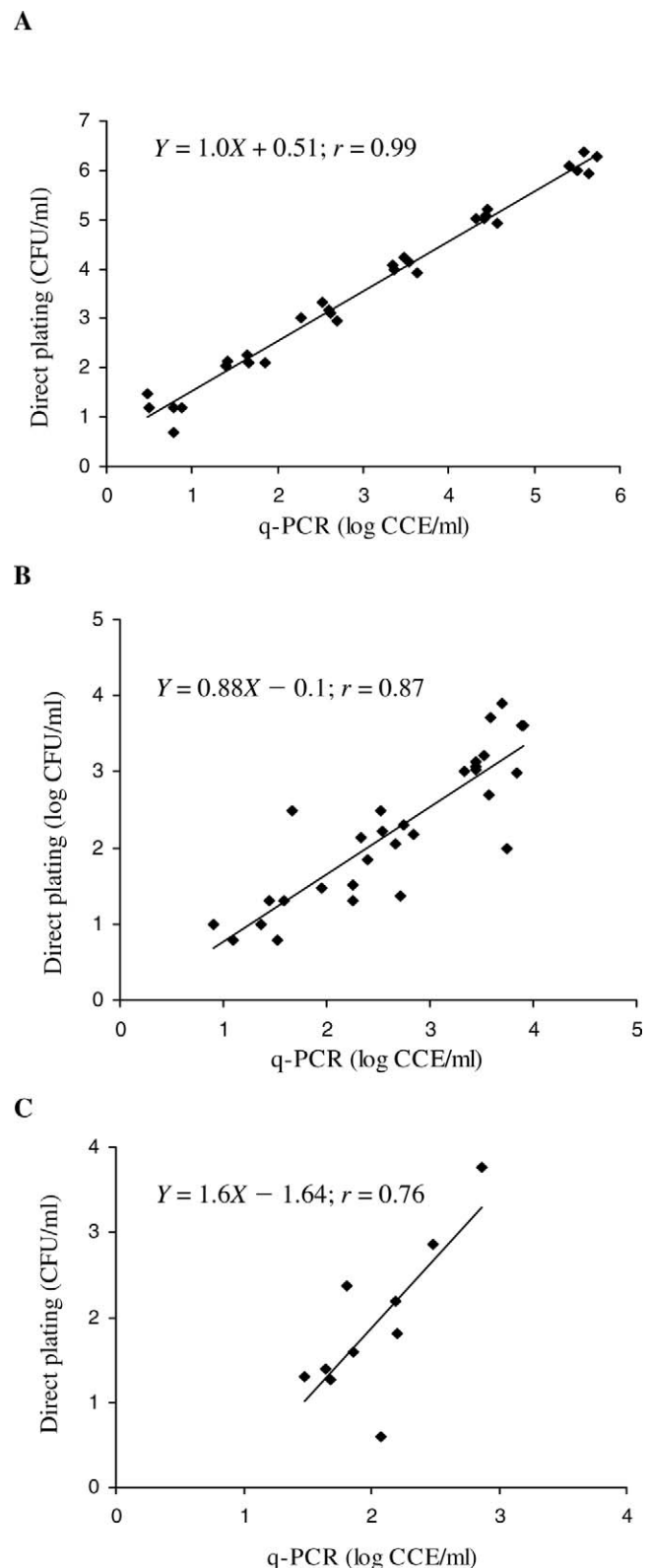


FIGURE 4. Correlation between direct plating and q-PCR results for enumeration of *C. jejuni* in spiked chicken rinse (A), on naturally contaminated carcasses from slaughterhouses, (B), and on carcasses from retail outlets (C).



direct plating with spiked chicken rinse was similar to that reported by Hein et al. (8), who quantified *Staphylococcus aureus* in spiked cheeses by q-PCR and direct plating. The regression line slope of 1.0 indicates that the efficiency of both the PCR and the DNA purification step is independent of the initial *Campylobacter* concentration. Direct plating consistently gave 0.5-log higher values than did the q-PCR, probably because of losses during the DNA extraction procedure and/or because the samples used for the standard curve contained more copies than calculated, leading to an underestimation of the target copies in the unknown samples. The counts of *C. jejuni* were on average the same for the two methods when naturally contaminated carcasses from slaughterhouses were analyzed. This indicates that these samples contained a higher proportion of dead and/or viable but not culturable cells (VNC) than did the spiked samples. The higher but presumably also more variable proportion of dead and/or VNC *Campylobacter* cells among the slaughterhouse samples may explain the lower correlation coefficient obtained for this set of samples. The carcasses from retail outlets were an even more heterogeneous group; the period between slaughter and analysis ranged from 3 to 9 days. The proportion of and variation in the number of dead and VNC *Campylobacter* cells were even higher in this group of samples, as indicated by a further decrease in the correlation coefficient. However, the correlation coefficient increased from 0.76 to 0.91 when the sample with the lowest correlation for the two methods was excluded. Samples with low plate counts are generally more likely to harbor a higher proportion of unculturable cells than are samples with high plate counts. This is presumably why the slope of the regression line for the retail samples was much steeper than that for the spiked samples.

A drawback of PCR methods is that DNA from dead bacteria also is counted, but techniques to circumvent this problem recently have been devised. Wolffs et al. (18) described the use of a discontinuous buoyant density gradient method to separate live from dead *Campylobacter* cells. The correlation between q-PCR and direct plating results was high, but the detection limit also was high, at 860 CFU ml<sup>-1</sup>. In several studies, ethidium monoazide bromide (EMA) has been used, which diffuses into dead cells. When the bacteria are then exposed to strong light, the EMA molecules are covalently attached to the DNA, preventing the *Taq* polymerase from amplifying the DNA. EMA treatment improved the correlation between direct plating and q-PCR results for total counts of slaughterhouse swabs (6). Methods quantifying only live bacteria will lose some sensitivity because of losses during the removal of dead cells. The additional separation step also may increase the cost, in terms of both analysis time and money. Depending on the application, differentiation between live and dead bacterial cells may not be critical, and as is shown in the present study, a high correlation between direct plating and q-PCR results can be obtained for carcasses analyzed shortly after slaughter.

A newly described real-time PCR method for quantitative detection of *S. aureus* in milk had sensitivities of 100 and 99% for bovine and caprine milk, respectively (7). A

sensitivity of 100% also was recorded for a real-time q-PCR method for detecting *Vibrio parahaemolyticus* in seafood (3). The q-PCR method developed in the present study failed to detect *Campylobacter* on 1 of the 14 naturally contaminated retail carcasses that tested positive with direct plating (sensitivity of 93%). However, for this sample only two colonies appeared on the large mCCDA plate where 1 ml of chicken rinse was spread and no growth was recorded on the plates where 100  $\mu$ l of chicken rinse was spread. All 30 carcasses from slaughterhouses that tested positive for *Campylobacter* with direct plating also tested positive with the q-PCR method, although four of the samples had counts between 6 and 10 CFU ml<sup>-1</sup>. Real-time q-PCR detected *Campylobacter* in 6 of the 31 samples from retail carcasses that were negative with direct plating (specificity of 81%, 25/[6 + 25]). In comparison, the methods for detecting *S. aureus* in bovine milk and *V. parahaemolyticus* in seafood had specificities of 12.5 and 91%, respectively (3, 7).

The impact on public health of a pathogen present on a specific food item can be estimated by a quantitative risk assessment. A prerequisite for such an assessment is accurate data on the concentration of the pathogen. The q-PCR method described here could be applied for obtaining quantitative data for risk assessments and for establishing a quantitative performance standard for *Campylobacter* in the poultry industry.

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