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Risk assessment of false-positive quantitative real-time PCR results in food, due to detection of DNA originating from dead cells

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Abstract

Real-time PCR technology is increasingly used for detection and quantification of pathogens in food samples. A main disadvantage of nucleic acid detection is the inability to distinguish between signals originating from viable cells and DNA released from dead cells. In order to gain knowledge concerning risks of false-positive results due to detection of DNA originating from dead cells, quantitative PCR (qPCR) was used to investigate the degradation kinetics of free DNA in four types of meat samples. Results showed that the fastest degradation rate was observed (1 log unit per 0.5 h) in chicken homogenate, whereas the slowest rate was observed in pork rinse (1 log unit per 120.5 h). Overall results indicated that degradation occurred faster in chicken samples than in pork samples and faster at higher temperatures. Based on these results, it was concluded that, especially in pork samples, there is a risk of false-positive PCR results. This was confirmed in a quantitative study on cell death and signal persistence over a period of 28 days, employing three different methods, i.e. viable counts, direct qPCR, and finally floatation, a recently developed discontinuous density centrifugation method, followed by qPCR. Results showed that direct qPCR resulted in an overestimation of up to 10 times of the amount of cells in the samples compared to viable counts, due to detection of DNA from dead cells. However, after using floatation prior to qPCR, results resembled the viable count data. This indicates that by using of floatation as a sample treatment step prior to qPCR, the risk of false-positive PCR results due to detection of dead cells, can be minimized.

Keywords: Real-time PCR; False-positive results; Dead cells; Yersinia enterocolitica; Floatation; Flotation

1. Introduction

Food-borne pathogens are recognized worldwide as a serious health threat (Wallace et al., 2000). Due to growing concerns, microbiological control programs are increasingly applied throughout the food chain to

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prevent food-borne disease (Malorny et al., 2003). It is of great importance that the analytical methods in such control programs are fast and reliable and it is advantageous if they can provide quantitative data. Traditional methods are commonly based on selective enrichment of the target pathogens (de Boer and Beumer, 1999). Even though these methods are standardized and efficient, they suffer from serious disadvantages, such as that they are time-consuming, correct analysis can be difficult due to lack of expression of phenotypic properties, and detection of viable-non-culturable (VNC) cells is impossible. In an effort to overcome these limitations, DNA-based detection methods have been developed (Scheu et al., 1998). One of the most promising methods is real-time PCR due to its speed, sensitivity, specificity and selectivity, high degree of automation and the possibility of target quantification (Jung et al., 2000). Real-time PCR is already used for rapid virus diagnostics (Mackay et al., 2002) and quantification of gene expression (Bustin, 2000), but is also becoming established in bacterial food-borne pathogen diagnostics.

One of the main challenges concerning correct pathogen diagnostics by real-time PCR lies in the nature of the method. Since real-time PCR detects nucleic acids, rather than living cells, there is a risk that nucleic acids originating from dead cells, will lead to positive PCR signals (Scheu et al., 1998). Until the development of real-time PCR and the opportunity to provide quantitative data arose, the risk of false-positive PCR results due to detection of dead cells was considered only a minor setback. The reason was that most PCR methods for food-borne pathogen diagnostics included an initial culture-enrichment step to improve sensitivity and after enrichment the amount of living cells considerably exceeded the amount of dead cells (Uyttendaele et al., 1999). However, methods such as culture-enrichment cannot be used prior to qPCR, since they influence the initial amount of target in an uncontrolled manner. This therefore, leads to a renewed interest in the risk of false-positive PCR results due to detection of dead cells.

Different studies during the last two decades have qualitatively determined the persistence of free DNA or DNA from dead cells and shown contradictory findings concerning the risk of false-positive PCR signals. Still, many studies showed extended persistence up to months, of PCR-detectable DNA in samples after death of target cells (Josephson et al., 1993; Allmann et al., 1995). A recent study by Nogva et al. (2000) used real-time PCR for quantitative assessment of degradation kinetics of pure cultures of *Campylobacter jejuni* and its DNA, in contrast to previous qualitative studies. The study showed that degradation of DNA could be rapid in some cases, such as after heat treatment of 100 °C when initial degradation was as fast as 4.5 log units in 6 h.

The aim of the present study was to evaluate, for the first time, the effect of complex food samples on the persistence of chromosomal and plasmid DNA. *Yersinia enterocolitica* was used as a model system. Furthermore, a more complex system of living, dying and dead cells in all stages of degradation, was studied with three different methods to assess the risk of false-positive PCR results in the case of direct quantification of the pathogen load with qPCR and in the case of application of a novel sample treatment called floatation prior to qPCR.

2. Materials and methods

2.1. Samples

Y. enterocolitica Y 79 was grown overnight in TSB (Tryptone Soy Broth, Oxoid CM129, Unipath, Basingstoke, UK) at 28 °C. CFU were determined using TGE agar plates (Tryptone Glucose meat Extract, Merck, Darmstadt, Germany) or, for specific *Yersinia* determination, CIN agar plates (Cefsulodin Irgasan Novobiocin; Yersinia selective agar base, Unipath). DNA was purified using Invitrogen EasyDNA kit (Invitrogen, Groningen, The Netherlands). The concentration of DNA was fluorimetrically determined using a TD-700 fluorimeter (Turner Designs, Sunnyvale, CA, USA) and the DNA was diluted to appropriate concentrations in sterile Millipore water. Pork and chicken were bought in a local supermarket. Rinse samples were made by adding 25 g of pork chop or chicken filet to 225 ml of physiological saline and mixing in a stomacher for 5 min. Afterwards the pork or chicken was removed from the sample. Homogenate samples were made by adding 25 g minced pork meat or minced chicken to 225 ml physiological saline and mixing in a stomacher for 5 min. Afterwards, the pork

or chicken meat remained in the sample. The absence of detectable *Y. enterocolitica* cells or its DNA in the food samples was confirmed by real-time PCR and selective plating. Dilution, 10- or 100-fold, of the food samples prior to PCR was made, when necessary, with physiological saline.

2.2. Real-time PCR

Two primer sets (Lantz et al., 1998) targeting a 0.3-kb part of the 16S rRNA gene from *Y. enterocolitica* and a 0.6-kb part of the plasmid-borne virulence gene *yadA* were used to develop a real-time PCR assay using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) (Wolffs et al., 2004a,b). The PCR mixture consisted of 0.75 U *Tth* DNA polymerase (Roche Diagnostics), 1× *Tth* DNA polymerase buffer (Roche Diagnostics), 4 mM of $MgCl_2$, 0.4 μM of each primer (one primer set used per assay), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 10,000 times diluted SYBR Green I (Roche Diagnostics) and 4 μl sample. The total volume was 20 μl. The use of *Tth* DNA polymerase was chosen due to its greater resistance to PCR inhibitors (Abu Al-Soud and Rådström, 1998; Lübeck et al., 2003) and, furthermore, its use also improves the amplification efficiency and widens the detection window (Wolffs et al., 2004a). Therefore, *Tth* DNA polymerase and its buffer were used for all experiments during this study. For purified DNA, each amplification started with a denaturation step of 1 min at 95 °C, followed by 40 cycles of 0.1 s denaturation at 95 °C, 5 s annealing at 58 °C and elongation for 25 s at 72 °C, followed by a single fluorescent measurement, and finally 25 s of final elongation. For samples containing whole cells, the protocol consisted of a denaturation step of 4 min at 95 °C, followed by 40 cycles of 10 s denaturation at 95 °C, 25 s annealing at 58 °C and elongation for 45 s at 72 °C, followed by a single fluorescent measurement, and finally 25 s of final elongation. Amplification was followed by a melting curve analysis between +65 and +95 °C, and finally a cooling step for 1 min at +40 °C. During amplification, the fluorescence was measured in channel F1, display mode F1.

For the primer set detecting the plasmid-borne virulence gene *yadA*, a new set of hybridization probes (PWY1: 5′-CTGTTGCCATTGGACACTC-TAGTCAC-fluorescein-3′; PWY2: 5′-LC red 640-TTGCGGCAAATCATGGTTATTC-3′) was developed. The PCR mixture consisted of 0.75 U *Tth* DNA polymerase (Roche Diagnostics), 1× *Tth* DNA polymerase buffer (Roche Diagnostics), 4 mM of $MgCl_2$, 0.4 μM of each primer (one primer set used per assay), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1 pmol of each probe (TIB MOLBIOL, Berlin, Germany) and 4 μl sample. The total volume was 20 μl. Each amplification started with a denaturation step of 1 min at 95 °C, followed by 40 cycles of 0.1-s denaturation at 95 °C, 10 s annealing at 58 °C followed by a single fluorescent measurement, and elongation for 25 s at 72 °C. Amplification was followed by a melting curve analysis between +50 and +95 °C, and finally a cooling step for 1 min at +40 °C. During amplification, the fluorescence was measured in channel F2/F1.

The quantification data, in terms of crossing point (Cp) values (Cp is expressed as a fractional cycle number and is the intersection of the log-linear fluorescence curve with a threshold crossing line), was determined using the second derivative method of the LightCycler Software, version 5.3 (Roche Diagnostics).

2.3. Detection window and amplification efficiency

Tenfold dilutions of *Y. enterocolitica* DNA between 1 mg/ml and 1 fg/ml or cells between 10^1 and 10^8 CFU/ml were used to obtain standard curves. All measurements were run in independent triplicate runs. After amplification, results from the melting curve were analyzed, and the Cp values of all samples that gave a positive specific product peak between 88 and 92 °C were plotted against the log of the initial DNA concentration. From this graph, the detection window was determined. After this, linear regression was used to calculate the slope of the Cp versus log initial DNA concentration plot using the points in the linear range. From this slope, the amplification efficiency was calculated using the following equation: $AE=(10^{-1/slope})-1$ (Klein et al., 1999).

2.4. Degradation of DNA in food

Degradation studies were performed by adding purified DNA to either chicken or pork samples (both rinsed and homogenized). The studies were performed

at two different temperatures: 4 °C (refrigerator) and 20 °C (approximate room temperature). To be able to follow the degradation correctly, standard curves of duplicate data were developed for the different samples at the correct dilutions to overcome possible PCR inhibition. The matching standard curves were then used to analyze the quantitative real-time PCR data. After plotting the log DNA concentrations versus time, linear regression was used to calculate the log degradation value (LDV), which is defined as the time of natural DNA degradation to obtain a DNA concentration reduction of 1 log unit.

$$\mathrm{LDV} = (t2 - t1)/(logN1 - logN2), \text{ in which}$$
$$t = \text{time and } N = \text{DNA concentration.}$$

2.5. Degradation of Y. enterocolitica cells in meat samples

Exponentially growing *Y.enterocolitica* cells were added to pork rinse or chicken rinse samples and maintained at 28 °C. Over a time period of 28 days, the presence of *Y. enterocolitica* was determined by viable counts, direct qPCR and floatation prior to qPCR. CIN agar plates were used for viable counts. Direct qPCR was performed by 10-fold dilution of the food samples in physiological saline, to allow qPCR measurements. Afterwards the samples were heated for 5 min at 95 °C to obtain sufficient cell lysis, after which 4 μl was added to the PCR mixture. Standard curves used for absolute quantification were made in appropriate 10-fold diluted food samples. For floatation prior to qPCR, a one-step floatation setup was used as described by Wolffs et al. (2004b).

In brief, three layers with different densities were carefully layered below each other. The bottom layer consisted of a high density solution mixed with the sample to a density of approximately 1.200 g/ml. The middle and top layers had densities of 1.087 and 1.065 g/ml. The resulting discontinuous gradients were centrifuged in a Sigma lab centrifuge (Sigma, Osterode am Harz, Germany) for 15 min at $4500\times g_{\max}$ in a swing-out rotor and afterwards 1 ml samples were taken, using sterile 2 ml syringes, for further analysis. The samples were added to 2 ml Eppendorf tubes, diluted with physiological saline to 2 ml (to obtain a density of the solution that allowed pelleting of cells) and centrifuged at $13{,}000\times g_{\max}$ in a benchtop Eppendorf centrifuge for 5 min. Afterwards, 1.75 ml of the supernatant was removed and the cells were resuspended in the remaining 0.25 ml after

Table 1
Standard curves generated for the two PCR assays in four different meat samples

PCR assay	Sample[a]	Standard curve equation[b]	R^{2c}	AE[d]	Detection window[e]
16S rRNA	Water	$y=-3.754x+7.400$	0.995	0.847	10^{-3}–10^{-9}
	Chicken rinse	$y=-3.652x+8.954$[f]	0.998	0.879	10^{-4}–10^{-9}
	Chicken homogenate	$y=-3.068x+10.77$[f]	0.945	1.118	10^{-4}–10^{-9}
	Pork rinse	$y=-4.799x+9.598$[f]	0.975	0.616	10^{-5}–10^{-8}
	Pork rinse[a1]	$y=-3.883x+8.364$	0.985	0.809	10^{-4}–10^{-8}
	Pork homogenate	$y=-3.451x+8.389$[f]	0.993	0.949	10^{-4}–10^{-7}
	Pork homogenate[a1]	$y=-3.721x+8.383$	0.994	0.857	10^{-3}–10^{-8}
yadA	Water	$y=-3.754x+7.40$	0.994	0.847	10^{-3}–10^{-9}
	Chicken rinse	$y=-3.159x+7.06$[f]	0.978	1.073	10^{-3}–10^{-8}
	Chicken homogenate	$y=-3.390x+8.87$[f]	0.860	0.972	10^{-3}–10^{-7}
	Pork rinse	$y=-4.435x+1.59$[f]	0.989	0.681	10^{-4}–10^{-8}
	Pork homogenate	$y=-4.064x+0.77$[f]	0.995	0.762	10^{-4}–10^{-7}

[a] All food samples were 10-fold diluted, except for the samples marked[a1], which are 100-fold diluted.
[b] Standard curves were created by plotting Cp value versus log DNA concentration. DNA concentrations from 1 mg/ml to 1 fg/ml were studied. Equations describing the data were obtained by linear regression.
[c] Square regression coefficient.
[d] Amplification efficiency.
[e] The detection window is defined as the range of DNA conc. (g/ml) for which Cp values and positive product peaks were obtained between 88 and 92 °C during melt curve analysis.
[f] Equations used in degradation studies and for generation of Figs. 1 and 2.

which they were heated for 5 min at 95 °C to obtain sufficient cell lysis. These final samples containing *Y. enterocolitica* cells were analyzed by qPCR. Standard curves for absolute quantification were made with cells diluted in physiological saline.

3. Results

3.1. DNA degradation in meat samples

Four different meat samples, i.e. chicken homogenate, chicken rinse, pork homogenate and pork rinse, were used as model systems to follow the degradation of DNA by using qPCR. Two different PCR assays for different *Y. enterocolitica* targets were used as models. Standard curves were established for the 16S rRNA (chromosomal DNA) assay and for the *yadA* (plasmid-borne DNA) assay (Table 1). Results showed that for most samples, with an increasing complexity, the slopes of the standard curves deviated more from the optimal slope of −3.32. Furthermore, when the complexity of the samples increased, the detection window became narrower. Finally, a new set of hybridization probes was designed for the *yadA* amplicon to improve the detection window, which was only 2 to 3 log units wide when SYBR Green was used (data not shown). When studying the DNA degradation (Figs. 1 and 2, Table 2), it was found that the degradation rate depended strongly on the type of sample. The fastest degradation was observed in chicken homogenate with both assays (LDV=0.5 and 1 h), while significantly slower rates were observed in pork samples (both assays at both temperatures giving LDV>35 h). The influence of temperature was also noticeable, with consistently faster degradation at 20 °C than at 4 °C.

3.2. False-positive PCR signals

In order to demonstrate the true risk of false-positive PCR results in the detection of *Y. enterocolitica* in meat

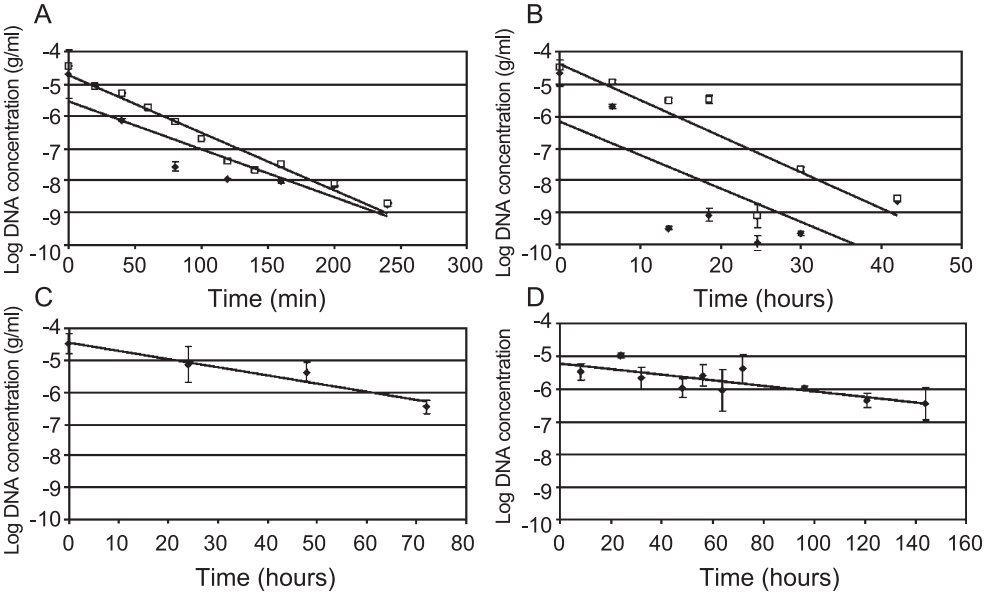

Fig. 1. Degradation of chromosomal *Y. enterocolitica* DNA in different food samples at different temperatures as measured by qPCR. (A) Chicken homogenate, (B) Chicken rinse, (C) Pork homogenate, (D) Pork rinse. □: 4 °C, ◆: 20 °C. Linear regression was used to generate equations describing the degradation kinetics in the samples. The following equations linear regressing models (time in h unless noted otherwise) were derived from the data: Chicken homogenate, 20 °C: $y=-0.0178x-4.7343$ (time in min), $.R^2$: 0.9576; Chicken homogenate, 4 °C: $y=-0.015x-5.5366$ (time in min), R^2: 0.8083; Chicken rinse, 20 °C: $y=-0.1125x-4.3613$, R^2: 0.7441; Chicken rinse, 4 °C: $y=-0.1036x-6.17$, R^2: 0.4957; Pork homogenate, 20 °C: $y=-0.0259x-4.4293$, R^2: 0.9429; Pork rinse, 20 °C: $y=-0.0083x-5.2371$, R^2: 0.6294.

Table 2
Log degradation values in four different meat samples and two different temperatures

PCR assay	Sample	Log degradation value (h)[a]
16S rRNA	Chicken homogenate, 20 °C	1.0
	Chicken homogenate, 4 °C	1.0
	Chicken rinse, 20 °C	8.0
	Chicken rinse, 4 °C	9.5
	Pork homogenate, 20 °C	38.5
	Pork rinse, 20 °C	120.5
yadA	Chicken homogenate, 20 °C	0.5
	Chicken homogenate, 4 °C	1.5
	Chicken rinse, 20 °C	0.5
	Chicken rinse, 4 °C	1.5
	Pork homogenate, 20 °C	74.5
	Pork homogenate, 4 °C	93.5
	Pork rinse, 20 °C	26.5
	Pork rinse, 4 °C	35.0

[a] Log DNA concentrations were followed and plotted versus time (h). Linear regression models were used to calculate the log degradation values representing the time it takes until 90% of the DNA is degraded.

samples, exponentially growing cells were added to pork rinse and chicken rinse and maintained at 28 °C. Due to the slower DNA degradation at lower temperature and due to possible preservation effects by a lower temperature, it was decided to perform the experiments at 28 °C to speed up cell death and DNA degradation. Over a time period of 28 days, the presence of the cells was checked with three methods: (i) viable counts, (ii) direct qPCR and (iii) floatation followed by qPCR. Results obtained from viable counts showed that, first of all, the death of the *Y. enterocolitica* cells follows different patterns in the two meat samples. In chicken rinse, a stationary phase was observed for 3 to 5 days, after which cell death occurred at a rate of 1 log unit per 7.6 days, whereas in pork rinse immediate but slower death set in, at a rate of 1 log unit per 10.2 days (Fig. 3A–B).

The generation of false-positive PCR signals after direct qPCR can be observed in both samples. Here again differences were seen between the two samples. In chicken rinse from day 7 and onwards, the data from dilution followed by qPCR showed a stable overestimation of the cell count of 0.43 log units. In the pork rinse samples, the overestimation of the cell count by direct qPCR gradually increased reaching 0.98 log units after 28 days. However, the estimates of cell counts using floatation followed by qPCR closely resembled those of the viable counts for both sample types, during the whole time period, with only a slight underestimation in chicken rinse of 0.08 log units.

4. Discussion

In order to systematically evaluate the risk of false-positive PCR signals caused by DNA originating from dead bacteria in food samples, it is necessary to study the degradation rates of nucleic acids in these samples. In this study, we applied qPCR to study DNA degradation kinetics in four food samples. The first step was preparation of standard curves for qPCR. As can be seen from the slopes of the standard curves deviating from the optimum of −3.32, PCR-inhibitory compounds were still present (Table 1) which is most likely caused by the meat samples, as meat samples have been shown to inhibit PCR in previous studies (Abu Al-Soud and Rådström, 1998; Lübeck et al., 2003). These data show clearly that it is necessary to prepare standard curves for the cells or DNA in the actual samples that are used since the standard curves for the same target differ in the actual food samples.

Studying the qPCR data, the results showed that DNA degradation rates varied between samples and the temperatures studied, with the fastest degradation at 20 °C in chicken homogenate for both chromosomal and plasmid DNA, and the slowest degradation for chromosomal DNA in pork rinse and for plasmid DNA in pork homogenate (Figs. 1 and 2). The finding that degradation depends on sample type and temperature confirms indications in previous studies. Kreader (1996) showed that an increase in temperature from 4 to 30 °C decreased PCR detectability of *Bacteroides distasonis* from 2 weeks to 1 day, where Romanowski et al. (1993) showed that differences in soil samples influenced the degradation of extracellular plasmid DNA. The results indicate, for example, that in case of low contamination levels in chicken rinse sample, it can be assumed that no false-positive results due to detection of dead cells will occur, whereas in pork samples there is a significant risk that false-positive results will show up. In order to be able to generate numerical data, linear regression was used on the obtained data to generate degradation models and equations. Using these equations, the log degradation

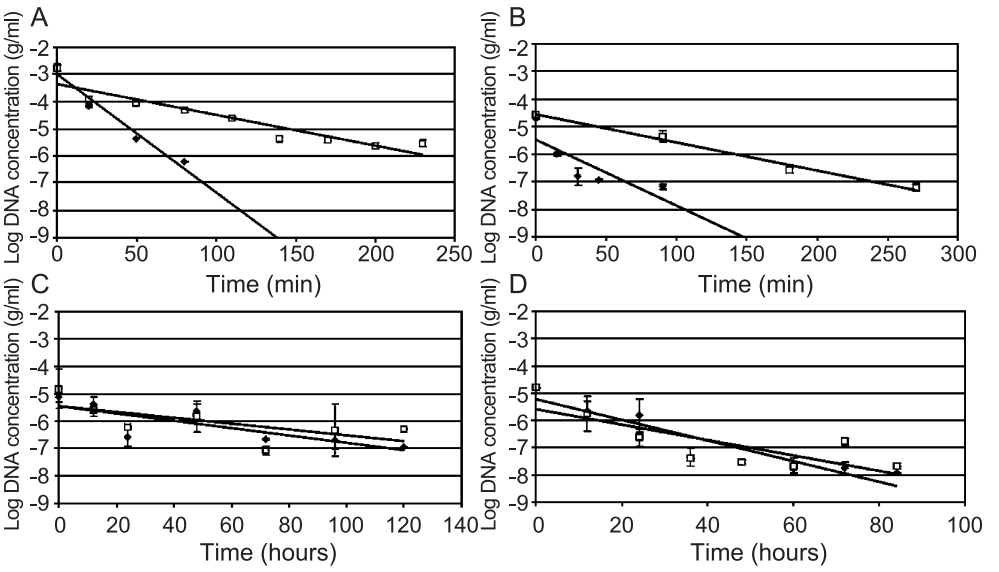

Fig. 2. Degradation of plasmid DNA from *Y. enterocolitica* in different food samples at 4 °C and 20 °C as measured by qPCR. (A) Chicken homogenate, (B) Chicken rinse, (C) Pork homogenate, (D) Pork rinse. □: 4 °C, ◆: 20 °C. Linear regression was used to generate equations describing the degradation kinetics in the samples. The following equations linear regressing models (time in h unless noted otherwise) were derived from the data: Chicken homogenate, 20 °C: $y=-0.0435x-2.9755$ (time in min), R^2: 0.9566; Chicken homogenate, 4 °C: $y=-0.0112x-3.3739$ (time in min), R^2: 0.8824; Chicken rinse, 20 °C: $y=-0.0239x-5.4679$ (time in min), R^2: 0.6655; Chicken rinse, 4 °C: $y=-0.0102x-4.559$ (time in min), R^2: 0.9845; Pork homogenate, 20 °C: $y=-0.0134x-5.4303$, R^2: 0.6598; Pork homogenate, 4 °C: $y=-0.0107x-5.4534$, R^2: 0.4607; Pork rinse, 20 °C: $y=-0.038x-5.2319$, R^2: 0.8593; Pork rinse, 4 °C: $y=-0.0284x-5.5864$, R^2: 0.6424.

value (LDV) was calculated in a similar manner as the log reduction value (*D* value) in preservation technology. In some cases, such as chicken homogenate at 20 °C, using the 16S rRNA assay, and chicken rinse at 4 °C using the *yadA* assay, the linear regression model showed good correlation (R^2>0.95), however, in some cases, a linear regression model could possibly be replaced in order to describe DNA degradation more accurately (for example, in pork homogenate at 4 °C with the *yadA* target, the correlation was very poor (R^2<0.50)). Nevertheless, the overall trends are clear and the degradation rates show that false-positive PCR signals may occur depending on the initial pathogen load and the time between microbial death and analysis.

The expected risk of false-positive results in pork rinse and chicken rinse was confirmed when direct qPCR was used to estimate numbers of CFU and compared to results for viable counts in the two samples (Fig. 3). It was found that cell death occurred slowly and the rate varied between the different samples (1 log unit per 7.6 days for chicken rinse and 1 log unit per 10.2 days in pork rinse). Slow cell death has previously been demonstrated for *Y. enterocolitica* in dried sausage (Kleemann and Bergann, 1996). Comparing the results from viable counts with those obtained with direct qPCR showed that after a period of cell death, overestimation was caused by the detection of DNA from dead cells in both samples. While the overestimation remained at a stable level in chicken rinse, in the case of pork rinse, the signal increased with time. This may be due to the very slow degradation rate of DNA in pork rinse (Table 2), which leads to a build-up of the amount of DNA originating from dead cells. Comparing the death rate, the rate in which the estimates generated by direct qPCR are generated with the previously described LDVs, it is clear that degradation of free purified DNA occurs faster than DNA originating from dead cells. A possible explanation of this may be partial release of DNA from dead cells and protection of the DNA by remains of the dead cells. This has been suggested by Kloos et al. (1994), saying that the microenvironment of DNA has different levels of

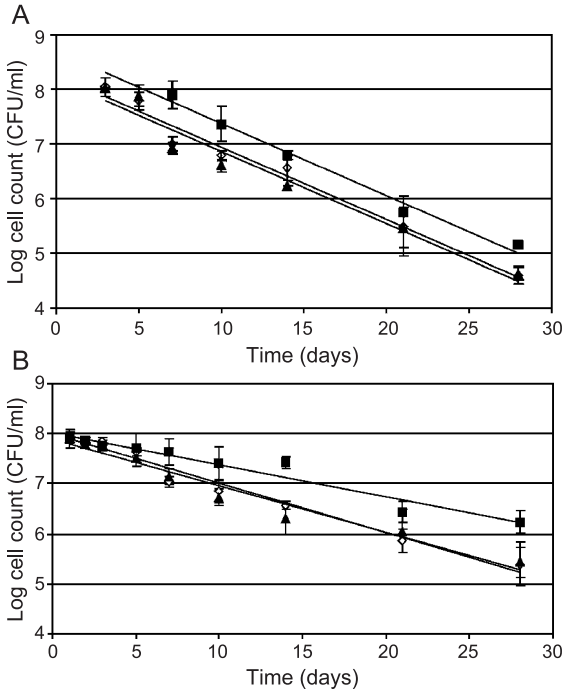

Fig. 3. Quantification of *Y. enterocolitica* cells in two food samples by viable counts, direct qPCR and floatation prior to qPCR. (A) Detection of *Y. enterocolitica* in chicken rinse samples. ◇: viable counts,: ■: direct qPCR (standard curve equation: $y=-3.59x+43.826$), ▲: floatation prior to qPCR (standard curve equation: $y=-3.34x+39.84$). Data from independent duplicate analysis. (B) Shows the detection of *Y. enterocolitica* in pork rinse samples. ◇: viable counts, ■: direct qPCR (standard curve equation: $y=-3.52x+43.509$), ▲: floatation prior to qPCR (standard curve equation: $y=-3.42x+40.311$). Data from independent duplicate analysis.

accessibility for macromolecules compared to free DNA. These findings imply that caution should be taken in studies where DNA degradation data are used to assess risks of false-positive data.

In order to deal with risks of false-positive PCR results, many researchers have investigated the use of mRNA as a viability marker, due to its rapid degradation compared to DNA (Alifano et al., 1994). However, mRNA studies have shown that the targeted genes have to be continuously expressed for correct quantification, and that the choice of mRNA extraction method and mRNA target influence the mRNA degradation rate (Sheridan et al., 1998; Norton and Batt, 1999). Another method that can be applied to prevent the risk of false-positive results due to detection of DNA from dead cells is immuno-magnetic beads (Kapperud et al., 1993; Nogva et al., 2000). However, where the beads can separate cell from free DNA, there are indications that non-viable bacteria with intact cell surface antigens can be enriched with immuno-magnetic separation (Hornes et al., 1991).

We have recently developed a new sample preparation method suitable for use prior to qPCR, called floatation (Wolffs et al., 2004a,b). Treating samples with this method based on buoyant density centrifugation allowed separation of free DNA from *Y. enterocolitica* cells, and amounts up to 2.1 μg/ml free DNA in samples did not lead to positive PCR results after floatation. This is because of the very low floatation rate of DNA compared to cells at the centrifugation force employed ($4500\times g$). Also, previous research on the density of dead *Y. enterocolitica* showed that a change in density took place after killing of the cells in different ways (Lindqvist et al., 1997). When comparing the results obtained through viable counts with those obtained when using floatation prior to qPCR, it was found that that the data resembled the viable count in contrast to direct qPCR, and therefore, the risk of false-positive PCR results was reduced. Future work will focus on further reducing the standard deviation in the floatation-PCR process by optimizing the target recovery, and the application of this method to the quantification of different targets in natural samples.

In conclusion, this study has for the first time provided quantitative data concerning DNA degradation kinetics in two types of pork and chicken samples. The risk of false-positive results was expected due to slow degradation rates, and indeed, monitoring cell death with viable counts and direct qPCR, confirmed that direct qPCR resulted in false-positive signals due to the detection of dead cells. A recently developed sample treatment method, floatation, was successfully applied prior to qPCR to circumvent the risk of false-positive results.

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