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Rapid Quantification of *Yersinia enterocolitica* in Pork Samples by a Novel Sample Preparation Method, Flotation, Prior to Real-Time PCR

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The development of real-time PCR thermal cycles in the late 1990s has opened up the possibility of accurate quantification of microorganisms in clinical, environmental, and food samples. However, a lack of suitable sample preparation methods that allow rapid quantification of the nucleic acids, remove PCR inhibitors, and prevent false-positive results due to DNA originating from dead cells has limited the use of quantitative PCR. We have used for the first time a new variant of density gradient centrifugation, called flotation, as a user-friendly sample preparation method prior to PCR. This paper describes the use of this sample preparation method, without DNA purification, for direct detection and quantification of *Yersinia enterocolitica* in PCR-inhibitory meat juice from pork. Flotation combined with qPCR could overcome PCR interference in juice from pork, as was shown by amplification efficiencies of 1.006 ± 0.021 and 1.007 ± 0.025, which are comparable to the amplification efficiency obtained for purified DNA samples (1.005 ± 0.059). Applying flotation to meat juice samples containing natural background flora and spiked with different levels of *Y. enterocolitica* showed that direct quantification of *Y. enterocolitica* was possible down to a level of at least 4.2×10^3 CFU per ml of meat juice, even in the presence of 10^6 CFU of background flora per ml. Finally, the results showed that samples containing large amounts of *Y. enterocolitica* DNA did not result in a positive PCR signal. This indicates that the risk of false-positive results due to detection of DNA originating from dead cells can be greatly reduced by using flotation prior to PCR.

Since real-time PCR thermal cycles became commercially available in the late 1990s, the technology has been recognized as an outstanding tool in molecular diagnostics (4, 8, 19). In comparison with conventional PCR, real-time PCR facilitates automation, computerization, and quantification of nucleic acids. Also, real-time PCR, with its increased sensitivity, speed, and precision and its wide dynamic range (2, 10), has found successful applications in food microbiology. Recently, numerous rapid real-time PCR assays for qualitative detection of pathogens have been developed (3, 13, 14). To allow such detection, several requirements must be met during sample preparation: (i) PCR-inhibitory substances must be removed, (ii) target nucleic acids or cells must be concentrated, (iii) heterogeneous samples must be converted into a homogeneous sample (25), and (iv) detection of dead cells must be prevented (10). Culture enrichment, DNA extraction, or a combination of both is often used to meet these goals (15). However, these steps are time-consuming, and their elimination can be regarded as one of the main challenges left on the way to the most rapid PCR detection possible.

Aside from the need for rapid qualitative detection, there is also a need for quantification of the bacterial load (6, 22). To allow quantitative measurements, one additional requirement for sample preparation is that the selected method should not influence the amount of target, or should do so only in a controlled and predictable manner. This means that enrichment cannot be used prior to quantitative PCR (qPCR). Concerning DNA purification, which is presently almost exclusively used, there may be some disadvantages. Studies have indicated that the quality and yield of DNA available after purification can depend on the purification method, sample composition (20), and target (29), and variations in both yield and quality can affect the final quantification data (22).

This study demonstrates the use of a novel sample preparation method, called flotation, based on density gradient centrifugation. The benefits of density gradient centrifugation as a sample pretreatment method are well established and include (i) the possibility of separating biological matrix particles and microorganisms with different buoyant densities (17); (ii) maintaining cell viability, which allows isolation and analysis of the microorganisms (24); and (iii) speed and easy handling. In contrast to sedimentation, after flotation the target is concentrated at the top of the density gradient, which has the advantage of reducing recontamination during recovery of the target.

The aim of this study was to develop a flotation-qPCR method that did not involve a DNA purification step for the rapid detection and quantification of *Yersinia enterocolitica* from pork juice using real-time PCR. Furthermore, the intention was to demonstrate the performance of flotation in the reduction of PCR inhibition, obtaining of homogeneous PCR samples from heterogeneous food samples, and separation of living cells from DNA originating from dead cells.

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TABLE 1. Buoyant densities of selected bacteria

Organism	Strain[a]	Buoyant density (g/ml)[b]
Acinetobacter calcoaceticus	SMRICC 66	1.070–1.075
Brochothrix thermosphacta	SMRICC 347	1.100–1.102
Hafnia alvei	SMRICC 370	1.070–1.080
Pseudomonas lundensis	SMRICC 456	1.050–1.055
Serratia liquefaciens	SMRICC 365	1.065–1.070
Y. enterocolitica	IHI 41526	1.084–1.090
Y. enterocolitica	Y79	1.076–1.081
Yersinia intermedia	Y344	1.087–1.090

[a] IHI, National Public Health Institute (KTL), Helsinki, Finland; SMRICC, Swedish Meats R&D Culture Collection, Lund, Sweden; Y, Norwegian School of Veterinary Science, Oslo, Norway.
[b] Average measurements of triplicate runs.

MATERIALS AND METHODS

PCR samples. Strains of *Yersinia* and background flora (BGF) (Table 1) were grown overnight in tryptone soy broth (Oxoid CM129; Unipath, Basingstoke, United Kingdom) at 28°C. CFU were counted using tryptone-glucose-meat extract agar plates (Merck, Darmstadt, Germany) or, for specific *Yersinia* determination, cefsulodin-irgasan-novobiocin agar plates (Yersinia Selective Agar Base; Unipath). DNA was purified using an EasyDNA kit (Invitrogen, Groningen, The Netherlands). The concentration of DNA was fluorimetrically determined using a TD-700 fluorimeter (Turner Designs, Sunnyvale, Calif.), and the DNA was diluted to appropriate concentrations in sterile Millipore water. Minced pork meat was bought at a local store, and meat juice collectors (Sarstedt, Nümbrecht, Germany) were filled with the meat and subjected to cycles of repeated freezing and thawing (between three and six cycles) to release the juice. Undefined BGF from pork was grown by inoculating 50 ml of tryptone soy broth with 5 g of minced pork and incubating it overnight. Pig feces were acquired from a local slaughterhouse and homogenized as described before. Human blood was withdrawn from a healthy subject, and both feces and blood samples were frozen before use.

Buoyant density centrifugation. Throughout the study, three different colloidal density gradient media, all formulated from RediGrad (Amersham Biosciences AB, Uppsala, Sweden) were used: BactXtractor-L (BX-L; density, 1.058 g/ml; pH 7.5; osmolality, 322 mosmol/kg), BactXtractor-M (BX-M; density, 1.132 g/ml; pH 7.5; osmolality, 300 mosmol/kg), and BactXtractor-H (BX-H; density, 1.309 g/ml; pH 7.5; osmolality, 340 mosmol/kg) (QRAB, Bålsta, Sweden). For all three media, the pH was adjusted with 1 M HCl and the osmolality was adjusted by the addition of ultrapure (99.99%) NaCl, using a Roebling osmometer (Labex Instruments AB, Helsingborg, Sweden). The final densities of BX-L and BX-H were reached after dilution and concentration of RediGrad. After the pH and the osmolality had been set, BX-M had a density of 1.132 g/ml and needed no further adjustment. The densities were measured using a DMA46 density meter (Instrument AB Lambda, Stockholm, Sweden).

The buoyant densities of microorganisms and food particles were determined as described by Pertoft (24). Briefly, 6 ml of BX-M was mixed with 2 ml of sample, 2 ml of physiological saline, and 50 μl of a density marker bead (DMB) solution (Amersham Biosciences AB) in a plastic 15-ml conical tube. Centrifugation was performed in a Braun-Sigma 4-15C laboratory centrifuge (Sigma, Osterode am Harz, Germany) at 10,000 × g_{avg} (average *g* force) for 30 min at room temperature, using a fixed-angle rotor (12170-H) for the 15-ml tube. A plot describing the densities of different locations in the self-generating gradient was made by measuring the distance from the bottom of the tube to the different layers of DMBs and plotting the known densities of the different DMBs (in grams per milliliter) versus distance (in centimeters).

Flotation. Based on the buoyant densities determined for microorganisms and food sample particles, appropriate flotation setups can be designed. A flotation setup was composed of layers of density gradient solutions, each layer with a different density. The goal of flotation is to separate and concentrate the target microorganism from the food matrix and the BGF. To achieve accumulation of the target, it is concentrated at the interface of two layers by choosing a top layer with a lower density than the target and a bottom layer with a higher density than the target. The opposite strategy can be adopted for the BGF and the food sample matrix. By choosing as narrow a density window as practically possible, the target will be separated from most of the BGF and the food matrix.

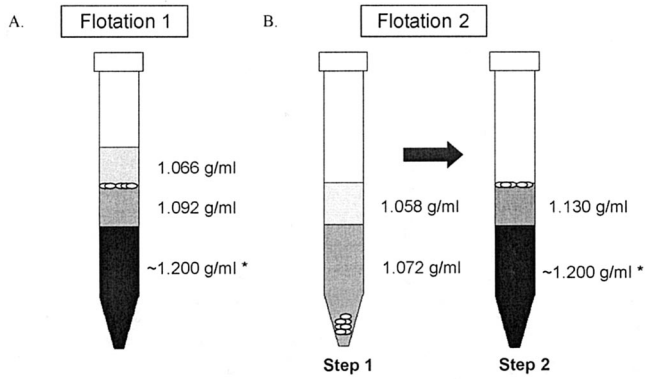

FIG. 1. Overview of the two flotation strategies investigated. (A) One-step flotation, consisting of three layers. The *Yersinia* recovery location (the location of *Yersinia* cells after centrifugation) is at the interface of the two top layers. The buoyant densities of the different colloidal solutions are shown. (B) Two-step flotation. Both steps contain two-layer flotations. During step 1, *Yersinia* concentrates at the bottom of the tube and is recovered from there to be used in step 2. The *Yersinia* recovery location in step 2 is on top of the top layer. *, layer composed of 3 ml of BX-H solution mixed with 1 ml of sample, resulting in a density of ~1.200 g/ml.

Experimental setup for flotation-qPCR. The flotation-qPCR procedure uses flotation 1 (Fig. 1) and takes place in the following seven steps. (Step 1) The sample is mixed with BX-H (1:3) and is loaded into a clean tube to form the bottom layer of the discontinuous gradient. (Step 2) The middle and top layers are carefully layered on top of the bottom layer. (Step 3) The discontinuous gradient is centrifuged for 15 min at 4,500 × g_{max} (maximum *g* force) in a swinging-bucket rotor. (Step 4) Samples (1 ml) are taken from the interface, using sterile 2-ml syringes. (Step 5) The samples are added to 2-ml tubes, diluted with physiological saline to 2 ml (to obtain a density of the solution that allows pelleting of cells), and centrifuged at 13,000 × g_{max} in a benchtop centrifuge for 5 min. (Step 6) A fivefold concentration step is performed by removing 1.8 ml of the supernatant and resuspending the cells in the remaining 0.2-ml samples containing *Y. enterocolitica* cells. (Step 7) Finally, the samples are analyzed by real-time PCR and viable counts are obtained.

Real-time PCR conditions. A primer set targeting a 0.3-kb part of the 16S rRNA gene from *Y. enterocolitica* (16) was used to develop a real-time PCR assay using the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) (28). The PCR mixture consisted of 0.75 U of *Tth* DNA polymerase (Roche Diagnostics); 1× *Tth* DNA polymerase buffer (Roche Diagnostics); 4 mM $MgCl_2$; 0.4 μM each primer; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; 10,000-fold-diluted SYBR Green I (Roche Diagnostics); and 4 μl of sample. The total volume was 20 μl. For purified DNA, each amplification started with a denaturation step of 1 min at 95°C followed by 40 cycles of 0.1 s of denaturation at 95°C, 5 s of annealing at 60°C, and elongation for 15 s at 72°C, and finally a single fluorescence measurement and 25 s of final elongation. For samples containing whole cells, prior to real-time PCR, the samples were heated for 5 min at 95°C to enhance cell lysis, and during PCR, three additional minutes of initial denaturation and 10 s of denaturation per cycle were added to obtain sufficient cell lysis. Amplification was followed by a melting curve analysis between +65°C and +95°C, and finally, a cooling step for 1 min at +40°C. During amplification, the fluorescence was measured in channel F1 and display mode F1. The quantification data, in terms of crossing-point (Cp) values, expressed as fractional cycle numbers and representing the intersection of the log-linear fluorescence curve with a threshold crossing line, were determined by using the second-derivative method of the LightCycler Software, version 5.3 (Roche Diagnostics).

Linear range of amplification and amplification efficiency. Tenfold dilutions of *Y. enterocolitica* DNA between 1 mg/ml and 1 fg/ml, or of cell cultures between 10^8 and 10^0 CFU/ml, were used to obtain standard curves. All measurements were run in independent triplicate runs. After amplification, the 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 or cell concentration. From this graph, the linear range was determined. After determination of the linear range of amplification, linear regression

was used to calculate the slope of the Cp-versus-log initial DNA or cell concentration plot using the points in the linear range. From this slope, the amplification efficiency (E) was calculated using the following equation: $E = (10^{-1/\text{slope}}) - 1$ (11).

PCR inhibition studies. To establish the reduction in PCR inhibition after flotation, runs using two different flotation setups containing meat juice with its natural BGF but lacking *Y. enterocolitica* were made. After flotation, samples were collected at the *Yersinia* recovery locations. By spiking these samples with a dilution series of *Y. enterocolitica* DNA and applying real-time PCR, the PCR efficiency in the samples was determined. This was compared to analysis of the same DNA dilution series added to untreated meat juice and pure water. Also, to test for the general applicability of flotation as a sample preparation method, two different matrices, pig feces homogenate and human blood, were run in the same manner.

RESULTS

Flotation. There are three main components in the food samples that ideally need to be separated from each other by flotation: (i) the target organism, *Y. enterocolitica*; (ii) the BGF; and (iii) the meat juice sample components. In order to perform flotation, the buoyant densities of *Y. enterocolitica*, several microorganisms found in pork (Table 1), and undefined BGF grown from pork and pork matrix were determined. Analysis of these three components in different tubes with self-generating gradients showed that the buoyant densities of these components differ. *Y. enterocolitica* Y79 (the model strain used for testing the flotation setups) was found to have a buoyant density of 1.076 to 1.081, and different populations of undefined BGF were found to have buoyant densities in the ranges of 1.062 to 1.065, 1.074 to 1.076, and 1.102 to 1.110. Finally, the meat juice sample contents concentrated at different buoyant densities of 1.066 to 1.070 and <1.018. The components of the meat juice which give the sample a red color, presumably hemoglobin and myoglobin, did not form a band in the density gradient. Rather, the color had the same distribution before and after centrifugation.

Based on the buoyant densities obtained for BGF, meat juice, and *Y. enterocolitica*, two flotation setups were designed (Fig. 1). Both were intended to exclude as much of the meat juice sample components and the BGF as possible from the *Yersinia* recovery location. One setup (flotation 1) allowed separation in one step, and the other (flotation 2) employed two successive centrifugation steps. The two-step procedure allowed easy sampling with less chance of recontamination of the recovered cells, since the target microorganism was recovered at the top of the density gradient after step 2. Running samples containing all three components in both setups showed that the red-colored fraction of the meat juice remained in the bottom layer, where the sample was loaded. Part of the BGF and the fractions of the meat juice with the lowest buoyant densities were found on the top of the density gradient in flotation 1 and were removed after the first centrifugation step in flotation 2. The fractions of the BGF and the meat juice with the highest buoyant densities were mainly found at the lower interface in flotation 1 and after the second centrifugation step in flotation 2. In the first setup, the *Yersinia* recovery location was at the interface of the top two layers. In the second setup, *Y. enterocolitica* was recovered from the top of the upper layer after the second centrifugation step.

PCR inhibition studies. When PCR mixtures containing untreated meat juice (20% [vol/vol]) were run, a solid white precipitate was formed in the LightCycler capillaries that totally prohibited fluorescence measurements (Fig. 2). Dilution of the samples to concentrations of 2, 0.2, and 0.02% (vol/vol) showed that only at 0.02% could the formation of precipitate be avoided, making real-time PCR measurements possible.

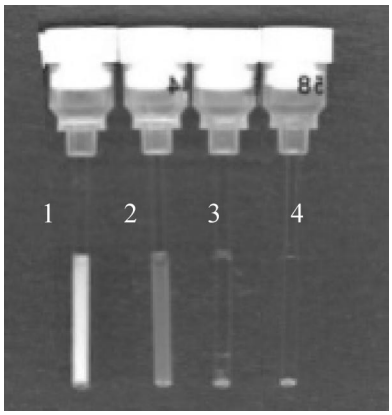

FIG. 2. Precipitate formation in LightCycler capillaries after PCR analysis of *Y. enterocolitica* in samples containing different concentrations of meat juice from pork. 1, 20% (vol/vol) meat juice; 2, 2% (vol/vol) meat juice; 3, 0.2% (vol/vol) meat juice; 4, 0.02% (vol/vol) meat juice.

The amplification efficiency obtained after qPCR of diluted meat juice samples containing *Y. enterocolitica* DNA was compared to the amplification efficiencies obtained from the relevant fractions (i.e., fractions recovered from the *Yersinia* recovery location) of undiluted meat juice samples subjected to the one- and two-step flotation protocols. The results showed that improved efficiencies could be obtained for the undiluted meat juice subjected to flotation, even compared to 1,000-fold-diluted meat juice without flotation (Table 2). The efficiencies of ~0.950 obtained for the flotation-treated samples, however, still deviate more from 1.000 than the value for purified DNA (1.005). To overcome this, the annealing step and/or the elongation step was extended by 20 s. The results show that amplification efficiencies can be increased to values of ~1.007 and

TABLE 2. Effects of different pre-PCR processing strategies on amplification efficiency

Sample	Treatment	Amplification efficiency[a]
Millipore water		1.005 ± 0.059
Juice from pork	Dilution (1,000-fold)	0.773 ± 0.031
	Flotation 1	0.954 ± 0.053
	Flotation 2	0.946 ± 0.037
	Flotation 1[b]	1.037 ± 0.064
	Flotation 1[c]	0.964 ± 0.048
	Flotation 1[d]	1.007 ± 0.025
	Flotation 2[d]	1.006 ± 0.021
Pig feces homogenate	Flotation 1[d]	0.991 ± 0.030
Human blood	Flotation 1[d]	1.030 ± 0.026

[a] Amplification efficiencies of *Y. enterocolitica* Y79 DNA in different samples. Efficiencies were calculated from data generated from three independent runs of purified *Y. enterocolitica* Y79 DNA in the sample.
[b] PCR protocol was extended with additional 20 s of annealing.
[c] PCR protocol was extended with additional 20 s of elongation.
[d] PCR protocol was extended with additional 20 s of annealing and 20 s of elongation.

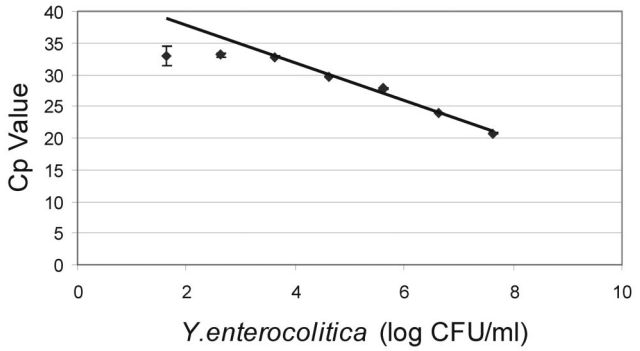

FIG. 3. Standard curve for absolute quantification of *Y. enterocolitica* cells. The linear range of amplification is shown by a line and can be described by the following formula: $y = -2.99 \times +43.826$; $R^2 = 0.9886$. The standard curve is a result of triplicate analyses, and all points shown were confirmed by melting curve analysis. The error bars indicate standard deviations.

1.006 for the one-step and two-step flotation setups, respectively. Finally, applying two very different sample types, such as blood and feces, showed that a reduction in inhibition to the same level could be obtained.

Flotation-qPCR. The possibility of directly detecting different amounts of *Y. enterocolitica* from meat juice harboring its natural BGF was studied using flotation setup 1. A standard curve was constructed to allow absolute qPCR (Fig. 3), and it showed a linear range of amplification between 5×10^7 and 5×10^3 CFU/ml. The results from viable counts and qPCR showed that after flotation the target was recovered in all cases, and there was good concurrence between viable-count and qPCR data (Table 3). Standard deviations for all measurements except six points were below 20%. Of those six points, five involved viable counts. The results also show that there is no obvious difference in the number of recovered *Y. enterocolitica* cells when the complexity of the sample is increased from saline (recovery, 34.5% ± 11.1%) by the addition of meat juice (recovery, 34.3% ± 6.0%) or additional meat juice and BGF (recovery, 31.9% ± 8.2%). For the two lowest concentrations, no quantitative data were obtained, since these concentrations fall outside the linear range of amplification. However, it was possible to confirm the presence of *Y. enterocolitica* cells.

Finally, to test whether DNA originating from dead cells can constitute a risk of false-positive signals in this method, *Y. enterocolitica* DNA samples were subjected to flotation 1. The results showed that after flotation, levels of up to 2.1 μg of chromosomal DNA/ml could not be recovered from the *Yersinia* recovery location. Before and after flotation, samples were taken from the bottom layer, i.e., the layer that constitutes the sample, and qPCR showed that similar amounts of DNA were found before and after flotation (data not shown), confirming that the DNA from dead cells does not float to the *Yersinia* recovery location.

DISCUSSION

The removal or neutralization of PCR inhibitors is one of the most important aspects of sample preparation (25). Because of the complex nature of biological samples and the differences in composition and content of PCR-inhibitory compounds, sample treatment often has to be optimized and chosen to suit each application and sample type (23). Although DNA extraction has several disadvantages, such as possible copurification of DNA from dead cells and yields varying with sample composition (20) and target (29), it is nonetheless, to our knowledge, exclusively used for qPCR detection of microbial pathogens from complex samples. This is mainly due to its high capacity to remove PCR inhibitors and deliver the clean sample needed for accurate quantification (21). The results showed that at least three different highly PCR-inhibitory (1) samples, such as meat juice, pork feces homogenate, and human blood, all showed amplification efficiencies in the same range as those for purified DNA in water and had a maximal deviation of 2.1% from the optimal amplification efficiency of 1.00 (100%) (Table 2). These results imply that the reduction of PCR inhibition is comparable to that of DNA purification with a commercial kit. It also indicates that inhibition caused by samples as different as meat juice, human blood, and pork feces homogenate can be reduced to similar levels by the same flotation protocol. The suitability of this method for different targets (different *Y. enterocolitica* strains and physiological states) and different samples depends largely on the design of the flotation layers. Furthermore, as long as buoyant density data for the target and the matrix are available, flotation setups

TABLE 3. Direct detection of Y. enterocolitica in juice from pork

Total no. of CFU of *Y. enterocolitica* added to sample prior to flotation	No. of CFU in 200 μl of *Y. enterocolitica* as determined by VCs and flotation combined with qPCR[a]					
	Saline		Meat juice		Meat juice + 10^6 CFU of BGF	
	VC	qPCR	VC	qPCR	VC	qPCR
3.0×10^1	0.8×10^1	+,+[b]	1.0×10^{1}*	+,−[b]	0.8×10^1	+,−[b]
4.1×10^2	1.4×10^{2}*	+,+[b]	1.3×10^2	+,+[b]	1.2×10^2	+,+[b]
4.2×10^3	1.3×10^3	1.4×10^3	1.5×10^3	1.4×10^3	1.7×10^3	1.5×10^3
4.1×10^4	0.8×10^{4}*	1.7×10^4	0.9×10^{4}*	1.7×10^4	0.5×10^{4}*	1.5×10^4
5.7×10^5	1.7×10^5	1.9×10^5	2.1×10^5	2.5×10^5	1.9×10^5	1.7×10^5
4.1×10^6	2.7×10^6	1.4×10^{6}*	1.7×10^6	1.2×10^6	1.3×10^6	1.9×10^6
5.7×10^7	1.7×10^7	1.8×10^7	1.9×10^7	1.8×10^7	1.7×10^7	1.8×10^7

[a] All results represent average data from at least duplicate analyses. Real-time quantitative data were obtained from a standard curve of *Y. enterocolitica* cells (Fig. 3). Standard deviations were in all cases between 0 and 20%, except for cases marked with an asterisk, which had standard deviations between 21 and 63%. VC, viable count.

[b] No quantitative data were available, since these points fall outside the linear range of amplification. However, positive signals confirmed by melting curve analysis are shown as pluses, and the absence of a positive signal is indicated by minuses.

can also be designed, optimized, and adjusted to suit other microorganisms or combinations of them. The buoyant densities determined for *Y. enterocolitica* in this study were close to previously published data (mean difference, <0.020 g/liter) (18), and the differences can be explained by differences occurring between strains, as has previously been shown for *Escherichia coli* strains (17). Furthermore, the use of different media for density gradient preparation has also been shown to be the cause of differences in the final buoyant density (5). By making sure that the middle layer is significantly heavier, and the top layer significantly lighter, than the known buoyant density of *Y. enterocolitica*, a safety margin can be built into the system, preventing loss of *Y. enterocolitica* to other locations than the *Yersinia* recovery location.

In a previous study, it was shown that qPCR can be improved by the use of alternative DNA polymerases, such as *Tth* (28). By using this DNA polymerase and its buffer in flotation-qPCR, quantification was possible over a range of at least 5 log units (Fig. 3). The results showed, furthermore, that the flotation-qPCR setup designed in this study resulted in accurate quantification of cells without a DNA purification step. No significant differences between qPCR and viable-count results, and no obvious difference in the amount of target cells recovered, could be found when the complexity of the samples was increased by the addition of meat juice or extra BGF. This indicates again that heterogeneous samples are turned into homogeneous samples after flotation treatment. The results also showed that the average recovery of cells after this flotation-qPCR process was 33.5% ± 8.5%. The recovery step is currently mainly limited and affected by manual handling. Future automation of sample recovery can improve the standardization of the process. Aside from this, it is possible to vary the degree of concentration by increasing the initial sample volume or adjusting the recovery volume of the final centrifugation after flotation. The present protocol incorporates a fivefold concentration step. By concentrating the sample after flotation, quantification can be enhanced.

Aside from qPCR results over a range of 5 log units, also outside the linear range of amplification, detection is possible. Due to interference from detection of primer dimers by SYBR Green, and due to detection probability issues at very low concentrations, Cp values were lower than could be expected and fell outside the linear range of amplification. Nonetheless, 100% qualitative detection outside this range was possible down to amounts of 4.1×10^2 CFU/ml, and can be considered to be semiquantitative. This information is still valuable, as it confirms the presence of the target in the sample. Therefore, apart from allowing quantitative measurements, these results show the improvement of the qualitative PCR method by combining it with flotation treatment, as previous studies showed that when 10^2 CFU of *Y. enterocolitica*/ml was combined with 10^6 CFU of BGF/ml, 100% detection was not possible, even after selective enrichment (12).

In food microbiology and the detection of food-borne pathogens, one of the major concerns about the use of a nucleic-acid-based detection method, such as PCR, has been the risk of false-positive results caused by the detection of DNA from dead cells (27). Our results showed that after flotation, free DNA remained separated from the *Yersinia* sampling location and that DNA concentrations of up to 2.1 μg/ml did not result in a positive PCR signal. This can be explained by the very low flotation speed of DNA at this centrifugation speed. Several other methods have been employed to overcome this risk. Enrichment is most commonly used prior to PCR, but the use of immunomagnetic beads, for example, should also allow the separation of living cells (9). However, there are indications that nonviable bacteria with intact cell surface antigens could be enriched by immunomagnetic separation (7). A study by Lindqvist et al. (18) indicated that the buoyant densities of *Y. enterocolitica* cells killed in four different ways differed from the buoyant density of living cells. Further studies will have to be performed to indicate whether such differences can be used in flotation to separate nonviable intact *Y. enterocolitica* cells from living cells.

One final aspect of importance for sample treatment methods is the time involved in the treatment. The seven steps involved in running one-step flotation PCR can be performed within 2 h, including 15 min of centrifugation and 1 h of real-time PCR analysis. This is comparable to rapid commercial DNA purification methods and significantly less time-consuming than traditional DNA extraction methods (26) or enrichment-PCR (12) in the case of qualitative measurement only.

In conclusion, this flotation-PCR method offers a rapid, easy combination of sample treatment and quantitative real-time PCR suitable for quantitative analysis of *Y. enterocolitica* in different types of samples within 2 h, without the risk of false-positive results due to DNA originating from dead cells.

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