

## Detection of *Salmonella* spp. in water using magnetic capture hybridization combined with PCR or real-time PCR

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### ABSTRACT

The removal of target DNA by magnetic capture hybridization (MCH) from constituents inhibitory to amplification by polymerase chain reaction (PCR) was evaluated using *Salmonella* as the test pathogen. Hybrids were subjected to both conventional and quantitative real-time PCR (qPCR). When PCR inhibitors commonly found in water were added to the reaction, MCH-PCR increased the detection sensitivity on the order of 8 to 2,000-fold compared with the system using only PCR. To determine the selectivity of MCH for target DNA (*Salmonella*), different amounts of non-target DNA (*Escherichia coli*) were added to the qPCR reaction. The highest non-target DNA concentration interfered with the amplification by qPCR alone, while MCH-qPCR was unaffected. Average recovery of *Salmonella* DNA by MCH-qPCR was 31% using optimized buffers, washing solutions and enzymatic digestion. A recovery function was proposed in order to calculate the real cell number based on the measured value. Preliminary testing confirmed the suitability of this method for analysis of natural waters.

**Key words** | inhibitors, magnetic beads, PCR, *Salmonella*, water

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### INTRODUCTION

Detection of pathogens using polymerase chain reaction (PCR) has become increasingly popular as a method of identifying low numbers of organisms in a variety of samples. Detection limits using molecular methods such as PCR may be lower when compared with conventional growth-based assays, and also have the advantage of increased specificity. Achieving low detection limits in any environmental pathogen assay is of paramount importance, especially in water samples where the presence of a single organism may result in human illness (Straub & Chandler 2003).

Successful PCR requires nucleic acid that is free from inhibitors and interfering compounds, and extraction protocols often dictate the success or failure of the goals of a particular assay. Wastewater and environmental samples may be physically and chemically complex, and are often poorly characterized. The application of PCR presents issues of recovery efficiencies of the pathogens under

study, and also awareness that the presence of inhibitors to enzymatic amplification in a reaction can increase detection levels above acceptable limits for human health with respect to recreational waters (Loge *et al.* 2002).

The list of known inhibitors of the PCR reaction is long and varied, and the concentration required to impede amplification is often quite low for some compounds (Wilson 1997). Samples from wastewater and water contain substances such as humic acids, metal ions and fats, which are potent inhibitors of PCR (Wilson 1997; Burtscher & Wuertz 2003). Methods to recover nucleic acids from these samples have been slow to develop and often result in the loss of material or are ineffective at removing compounds inhibitory to PCR (Harry *et al.* 1999). Clean-up methods include size-exclusion chromatography, electrophoresis, ion-exchange chromatography (Cullen & Hirsch 1998) and bispeptide nucleic acids (Chandler *et al.* 2000). The method of DNA purification must be carefully chosen with respect

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to the type of sample, and nucleic acid extraction protocols can also have an influence on the degree to which inhibitors are co-extracted and purified along with PCR template (Miller *et al.* 1999).

An alternative to conventional methods for purifying nucleic acid from inhibitors involves hybridization in solution with biotin-labelled oligonucleotide capture probes and magnetic beads coated with streptavidin. The strong affinity between biotin and streptavidin ( $K_D = 10^{-15}$  M) permits the separation of hybrid from non-target nucleic acid, interfering compounds and chemical species. This technique of combining magnetic capture hybridization with PCR (MCH-PCR) has been applied to pathogen detection in a wide variety of sample matrices, including plant material (Langrell & Barbara 2001), food (Chen & Griffiths 1998), air samples (Maher *et al.* 2001), clinical samples (Mangiapan *et al.* 1996), feces (Marsh *et al.* 2000) and bacteria in soil (Shapir *et al.* 2000). Many of these and other studies have demonstrated an improvement of PCR detection limits using the aforementioned beads.

To address the issues of efficiency, sensitivity and reliability in purifying nucleic acids from environmental samples, the purposes of this study were to (i) examine the qualitative impact that a range of inhibitors at various concentrations has on the detection of *Salmonella* DNA using MCH-PCR; and (ii) combine the techniques of magnetic capture hybridization with quantitative, real-time PCR (MCH-qPCR) to assess the efficiency of capturing *Salmonella* DNA.

## METHODS

### Bacterial cultures

Method development was done using *Salmonella enterica* serovar Typhimurium (ATCC 13311) as a model organism. Capture probe specificity to the *invA* gene was demonstrated using 10 strains of *Salmonella enterica*: serovar agona KS4, serovar give EI 1, serovar infantis FR, serovar infantis subsp. sensibel, serovar thompson, serovar Typhimurium, serovar Typhimurium b, serovar Typhimurium KS1, serovar Typhimurium KS2, serovar Typhimurium QB1. In addition, nine other strains were tested: *S. bongori*

DSM 13772, *S. cholerasuis* subsp. *arizonae* DSM 9386, *S. cholerasuis* subsp. *houtenae* DSM 9221, *S. cholerasuis* subsp. *salamae* DSM 9220, *S. isangi*, *S. livingstone*, *S. ohio* KS3, *S. rauhform* QB2 and *S. rissen*. All strains were cultured overnight at 37°C in 10 ml Luria-Bertani broth (Fisher Scientific). Cultures were centrifuged at 6,000 × g for 12 min, and resuspended in an appropriate volume of 1X TE buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.5).

### Cell enumeration and DNA extraction

Washed cells were fixed by addition of three volumes of 4% paraformaldehyde in 1X PBS (130 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2) and stored at 4°C for 2 h. Cells were washed by centrifuging at 6,000 × g for 12 min, resuspended in 1X TE buffer, and filtered through a 0.22-µm black polycarbonate filter. One hundred microlitres of 1 µg ml<sup>-1</sup> DAPI (4,6-diamidino-2-phenylindole) was added to the filter surface, which was stained for 10 min at room temperature. Filters were mounted in Citifluor and viewed using a Zeiss Axioskop with a 63X oil objective and a DAPI filter set (Omega Optical, Brattleboro, Vermont). A minimum of 500 cells were counted in duplicate and the average was used to calculate the original cell concentration. DNA was released from cells by heating in a 100°C water bath for 10 min and cooling on ice. Serial ten-fold dilutions were prepared using sterile double-distilled water.

Additionally, a comparison using mechanical and chemical lysis (FastDNA SPIN Kit for Soil, Qbiogene, Inc., Carlsbad, California) was performed according to manufacturer's recommendations.

### Primers and probes used for PCR and MCH

All primers and probes used were based on previously published test systems with the exception of the MCH capture probe, which was designed using standard sequence analysis software (Table 1).

### MCH-PCR

Three general steps are involved in the capture of DNA sequences and subsequent PCR amplification: (1) hybridization of target DNA with biotin-labelled probe(s); (2)

**Table 1** | Capture probe, PCR primers and internal probe used to detect *invA* gene in *Salmonella*

Oligonucleotide designation	Sequence (5'–3')	Position	Reference
INVA-1	ACAGTGCTCGTTTACGACCTGAAT	104–127	Chiu & Ou 1996
INVA-2	AGACGACTGGTACTGATCGATAAT	324–347	Chiu & Ou 1996
INT-CAP <sup>†</sup>	ATATCGTACTGGCGATATTGGTGTAT	205–242	This study
StyinvA-JHO-2-left	TCGTCATTCCATTACCTACC	167–186	Hoorfar <i>et al.</i> 2000
StyinvA-JHO-2-right	AAACGTTGAAAACTGAGGA	234–285	Hoorfar <i>et al.</i> 2000
Target probe	FAM-TCTGGTTGATTTCCTGATCGCA-TAMRAp	189–210	Hoorfar <i>et al.</i> 2000

<sup>†</sup>5' end labelled with biotin on a C6 spacer arm

binding of hybrid to streptavidin coated magnetic beads and separation of bead–hybrid complex from solution using a magnetic field; and (3) PCR amplification.

### Hybridization

The hybridization solution consisting of 200  $\mu$ l of hybridization buffer, 1.5 pmole INT-CAP, and 20  $\mu$ l of template DNA, was incubated at 50°C overnight with gentle end-over-end mixing in a hybridization oven (Boekel Scientific, Feasterville, Pennsylvania). Two different hybridization buffers were evaluated: 1X Binding and Washing Buffer (B&W) consisting of 5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 M NaCl (Dynal, Oslo, Norway) and 1X Hybridization Solution (Sigma, St Louis, Missouri).

### Binding and separation

M-280 Streptavidin coated magnetic beads (Dynal) were washed according to the manufacturer's recommendations and resuspended in 1X TE buffer. Ten microlitres of washed beads was added to the hybridization mixture, and incubated at 24°C with gentle mixing for 1 h. Tubes were placed in a magnetic stand (MPC-S, Dynal) and washed twice according to the manufacturer's specifications. The beads that were hybridized using the B&W buffer were washed using the same buffer, and the others with a 1X PBS/ 0.1% BSA solution (Marsh *et al.* 2000). Beads were resuspended in 40  $\mu$ l of dH<sub>2</sub>O.

### Conventional PCR

Five microlitres of hybridized beads were amplified by conventional PCR using a 50  $\mu$ l reaction volume consisting of 1X PCR buffer, 0.25 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 0.25 mM INVA-1, INVA-2, 1U Amplitaq Gold DNA polymerase (Applied Biosystems, Foster City, California). The reaction was carried out using a GeneAmp 9700 thermocycler (Applied Biosystems) with the following profile: initial denaturation 10 min at 95°C, then 35 cycles of 95°C for 20 s, 55°C for 30 s, 72°C for 30 s, and a final end extension step of 72°C for 7 min.

### Effect of inhibitors on conventional PCR and MCH-PCR detection of *Salmonella*

The following compounds and ions were used to assess their impact on PCR: humic acid (Sigma), 1.5  $\mu$ g  $\mu$ l<sup>-1</sup>, 1.0  $\mu$ g  $\mu$ l<sup>-1</sup>, 200 ng  $\mu$ l<sup>-1</sup>, 20 ng  $\mu$ l<sup>-1</sup>, 2 ng  $\mu$ l<sup>-1</sup>, 1 ng  $\mu$ l<sup>-1</sup>, 0.2 ng  $\mu$ l<sup>-1</sup>; Fe<sup>3+</sup>, 740  $\mu$ M, 74  $\mu$ M, 37  $\mu$ M, 3.7  $\mu$ M, 370 nM, 37 nM; Ca<sup>2+</sup>, 20 mM, 2 mM, 1 mM, 200  $\mu$ M, 100  $\mu$ M, 20  $\mu$ M, 2  $\mu$ M; triglycerides (Sigma), 300  $\mu$ g  $\mu$ l<sup>-1</sup>, 200  $\mu$ g  $\mu$ l<sup>-1</sup>, 140  $\mu$ g  $\mu$ l<sup>-1</sup>, 100  $\mu$ g  $\mu$ l<sup>-1</sup>, 40  $\mu$ g  $\mu$ l<sup>-1</sup>, 20  $\mu$ g  $\mu$ l<sup>-1</sup>; and aluminium (Cat-floc TL, Calgon, Pasadena, Texas), 0.12  $\mu$ g  $\mu$ l<sup>-1</sup>, 1.2  $\mu$ g  $\mu$ l<sup>-1</sup>, 12  $\mu$ g  $\mu$ l<sup>-1</sup>, 120  $\mu$ g  $\mu$ l<sup>-1</sup>, and 440  $\mu$ g  $\mu$ l<sup>-1</sup>. Stock solutions were prepared for each compound using sterile, double distilled water. To determine the minimum inhibitory concentration for conventional PCR, *Salmonella* DNA from 2,000 cells was mixed with varying concentrations of inhibitory compounds in the

PCR reaction. The PCR products were analysed on a microcapillary electrophoresis chip (Bioanalyzer 2100, Agilent, Palo Alto, California). To determine the inhibitor removal capacity of the magnetic beads, increasing concentrations of each compound were added to the hybridization mixture along with 20  $\mu\text{l}$  *Salmonella* DNA. The hybridization and bead binding was performed as described previously. Five microlitres of eluted DNA was amplified by conventional PCR and analysed using microcapillary electrophoresis.

### MCH-qPCR

Five serial ten-fold dilutions of nucleic acid from *Salmonella* cells were used to generate the standard curve for all MCH-qPCR determinations. Each dilution point in the standard curve was done in triplicate. Enumeration of cells and extraction of DNA from *Salmonella* was performed as described above. Twenty microlitres of DNA from the same dilutions used to generate the standard curve was added to the MCH tubes and the bead procedure was followed as described above. Five microlitres of hybridized beads was added to a 45  $\mu\text{l}$  reaction volume containing 1X TaqMan Master Mix, 900 nM Styinva-JHO-2-left and Styinva-JHO-2-right, and 200 nM target probe. Amplification was performed on a GeneAmp 5700 Sequence Detection System (Applied Biosystems) using the following thermocycle profile: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 60°C for 1 min.

Detection and quantification of amplified DNA was determined using SDS software provided by the manufacturer (Version 1.3 Applied Biosystems). All MCH-qPCR reactions were performed in triplicate. Positive controls included 5  $\mu\text{l}$  of DNA from each dilution to ensure accuracy of cell number added to MCH reaction. Negative controls were included in each assay.

### Restriction digest of *Salmonella* DNA

Short fragments of the *invA* gene were generated by incubating 20  $\mu\text{l}$  of *Salmonella* DNA with 10 units of the restriction enzyme Hph 1 in 80  $\mu\text{l}$  of NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9). Digestion was done at

37°C for 1 h, and the enzyme inactivated by heating at 65°C for 20 min. Duplicate reactions without enzyme were included in each experiment to compare MCH efficiencies using long and short fragments of DNA.

### Effect of non-target DNA on qPCR and MCH-qPCR

*Escherichia coli* (ATCC 15597) cells were cultured, harvested, counted, and the nucleic acid extracted using the methods outlined above. DNA from *Salmonella* (target) was mixed with *E. coli* (non-target) in the following cellular ratios: 1:100, 1:1,000, 1: 10,000, 1: 50,000. For *Salmonella*, DNA from a total of 250 cells was included in each reaction. For comparison, appropriate volumes of template were included in two reactions: MCH-qPCR and qPCR (TaqMan).

### Recovery function

To check the quality of results and the nature of the systematic deviation following MCH-qPCR, the data were analysed employing a statistical procedure. A recovery function, relating the original ( $x$ ) and the measured (from MCH-qPCR) cell numbers ( $x_m$ ), was established:

$$x_m = a_m + b_m x \quad (1)$$

where  $a_m$  and  $b_m$  are the origin ordinate and the slope, respectively.

The process standard deviation of the calibration function ( $S_{x_0}$ ) was calculated according to

$$S_{x_0} = \frac{S_y}{b} \quad (2)$$

where  $S_y$  is the residual standard deviation

$$S_y = \sqrt{\frac{\sum_{i=1}^N (y_i - \hat{y}_i)^2}{N - 2}} \quad (3)$$

and  $\hat{y}_i$  is the calculated cell number for the standard curve, defined by

$$\hat{y}_i = a + b x_i \quad (4)$$

with  $N$  the number of samples analysed,  $y_i$  the cell number

for each sample, and  $a$  (intercept) and  $b$  (slope) are the parameters for the calibration curve.

The standard deviation of the recovery function  $S_{ym}$  was calculated from the equation

$$S_{ym} = \sqrt{\frac{\sum_{i=1}^N [x_{m_i} - (a_m + b_m x_i)]^2}{N - 2}} \quad (5)$$

The process standard deviation of the calibration function ( $S_{xo}$ ) and the standard deviation of the recovery function for the MCH-qPCR ( $S_{ym}$ ) were tested for significant differences by defining the ratio

$$TV = \left(\frac{S_{ym}}{S_{xo}}\right)^2 \quad (6)$$

using the  $F$ -test: if  $TV > F(f_1 = f_2 = N - 2, P = 99\%)$ , then a significant difference between the standard deviations exists and it is necessary to find the cause of the high imprecision or to change the recovery function (Funk *et al.* 1995).

### Application of MCH-qPCR to a water sample

A local river was selected to test the proposed method. This location was chosen because the water was representative of turbid environmental samples likely to contain high concentrations of inhibitors. Fifty millilitres of sample was collected and centrifuged at  $5,000 \times g$  for 10 min to concentrate inhibitors and sediment. The pellet was resuspended in  $500 \mu\text{l}$  of 1X TE buffer and mixed with  $1 \times 10^6$  *Salmonella* cells. An additional control consisted of deionized water spiked with *Salmonella* cells. Nucleic acid was extracted and enzymatically digested as described above. An unspiked control was analysed to ensure the absence of any indigenous *Salmonella*. Both control and river sample were subjected to MCH-qPCR and qPCR as described above. All samples were analysed in duplicate.

## RESULTS AND DISCUSSION

### DNA extraction

Extraction of *Salmonella* DNA was evaluated comparing a simple heat extraction to bead-beating. The results from

bead-beating were variable and were at least 50% lower than for heat treatment.

### Specificity of capture probe

The biotin-labelled capture probe, INT-CAP, was tested against 19 strains of *Salmonella* by MCH-qPCR. All strains except two tested positive. DNA from *S. agona* and *S. cholerasuis* subsp. *houtenae* failed to hybridize (no cells detected) and also was not amplified when added directly to a qPCR reaction. *S. agona* is commonly isolated from warm-blooded animals; *S. cholerasuis* subsp. *houtenae* has been isolated from cold-blooded animals and rarely from humans (Brenner *et al.* 2000). Nearly all *Salmonella* strains represent a risk for human health, but the virulence varies depending on the strain and host characteristics.

### Effects of inhibitors on MCH-PCR

PCR inhibitory compounds known to be common to environmental and wastewater were spiked into PCR reactions containing constant concentrations of *Salmonella* DNA. Based on electrophoresis results, a minimum concentration was established for each compound that resulted in PCR inhibition in a standard reaction assay. For comparison, a duplicate assay was performed with the compounds added to the hybridization mixture along with *Salmonella* DNA, then subjected to MCH-PCR. Inhibitory concentrations were compared for the two assays.

For each compound tested, the MCH-PCR method was quite effective at removing DNA from the tested inhibitory constituents (Table 2). MCH was an efficient removal mechanism for humic acid in particular. Humic acids are ubiquitous in the environment; they comprise a very large, complex group of compounds, which have been shown to exert a variety of deleterious effects on amplification (Jacobsen 1995). Concentrations inhibiting PCR are dependent not only upon the source and purity, but also on the DNA polymerase used (Tebbe & Vahjen 1993). The minimum inhibitory concentration (MIC) for humic acid was increased by three orders of magnitude by employing the MCH procedure.

To allow a comparison between MICs in a standard PCR and the MCH-PCR, it was necessary to spike the

**Table 2** | Comparison of PCR inhibition in two different reaction systems

Compound	Minimum inhibitory concentration in PCR reaction		Increase in PCR sensitivity with MCH
	PCR	MCH-PCR	
Humic acid	> 0.20 ng $\mu\text{l}^{-1}$	> 200 ng $\mu\text{l}^{-1}$	1,000-fold
Calcium	> 20 $\mu\text{M}$	> 20 mM	1,000-fold
Iron	> 370 nM	> 740 $\mu\text{M}$	2,000-fold
Lipids	> 40 $\mu\text{g l}^{-1}$	> 300 $\mu\text{g l}^{-1}$	7.5-fold
Aluminium	> 1.2 $\mu\text{g l}^{-1}$	> 120 $\mu\text{g l}^{-1}$	100-fold

contaminant into the hybridization mixture. The compounds were assumed to have been removed following the washing procedure and, therefore, their concentrations were reduced in the PCR reaction. Alternatively, the compounds could have exerted inhibitory effects on the hybridization of *Salmonella* DNA to the labelled probes, or upon the binding of hybrids to the beads. The mechanism of inhibition in either PCR or MCH-PCR was not the objective of this study.

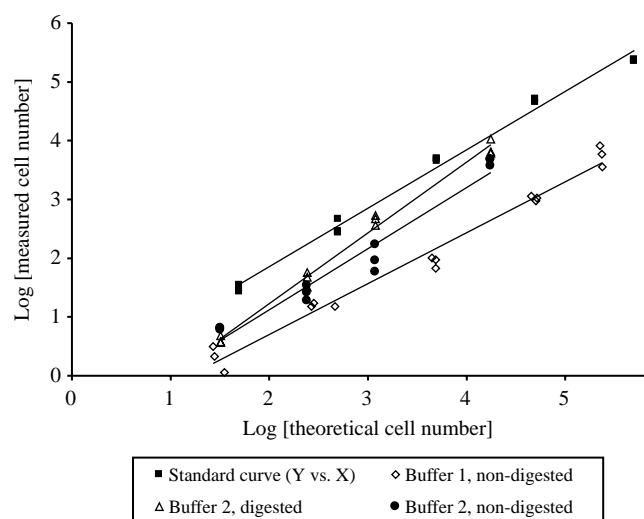
### Restriction digest of *Salmonella*

Hybridization of short fragments may be more efficient when binding hybrids to the beads due to minimization of steric effects. For this reason, enzymatic restriction of the *invA* gene was used to cleave the DNA near the region of capture and amplification. The results indicate that using shorter lengths of DNA for hybridization translates into higher recoveries, but only when the concentration of DNA is relatively high. Figure 1 shows the recovery of DNA after MCH-qPCR for both digested and undigested samples. For concentrations greater than 100 cells  $\mu\text{l}^{-1}$ , digestion of the DNA had a positive effect on the recovery of *Salmonella* in the range tested.

### Effect of non-target DNA on qPCR

The ability of MCH to recover target DNA from varying concentrations of non-target DNA was evaluated using qPCR. The results are presented in Table 3. Using only

qPCR, detection was reduced by one-fifth when the ratio of target to non-target was 1:50,000. For the concentrations tested, the recovery of *Salmonella* DNA using MCH-qPCR ranged from 42.3% to 48%, and was unaffected by high background levels of non-target DNA. Clearly, MCH can be an effective mechanism for isolating and removing target nucleic acid from matrices that may contain high amounts of interfering DNA. Such a complex matrix can be found in water samples, where pathogen concentrations may be quite low compared with relatively high levels of prokaryotic and eukaryotic organisms.



**Figure 1** | Detection after MCH-qPCR. Buffer 1: hybridization and washing with B&W buffer; buffer 2: hybridization buffer and PBS/BSA solution for washing.

**Table 3** | Effects of non-target DNA on quantitative TaqMan PCR assay in two reaction systems

Target: Non-target ratio <sup>†</sup>	qPCR‡	MCH qPCR‡
1: 100	270.55 ± 7.84	116.55 ± 26.77
1: 1,000	315.31 ± 33.05	105.83 ± 13.70
1: 10,000	275.42 ± 20.40	114.18 ± 17.20
1: 50,000	47.96 ± 1.95	120.15 ± 12.29

<sup>†</sup>1:100 represents 250 cells of *Salmonella* (target) to 25,000 cells of *E. coli* (non-target)

<sup>‡</sup>Calculated mean cell number of target detected

### MCH-qPCR recovery function

The results obtained from MCH-qPCR for serial ten-fold dilutions and expressed as cell numbers, revealed a systematic constant deviation for the undigested samples, independent of the template concentration, which resulted in a parallel displacement of the recovery curve in relation to the calibration curve (Figure 1). That displacement was greater for the sample that was hybridized and washed with B&W buffer (buffer 1), giving low recoveries of DNA between 2 and 5%. In contrast, when using hybridization buffer and PBS/BSA solution for washing (buffer 2), recoveries increased to 20–30%. The results for the digested sample (buffer 2) demonstrated a proportional systematic deviation, which was dependent on the concentration of the cells. In this case the recoveries varied from 12% for the lower cell numbers to 50% for the higher cell levels. The parameters for all the curves and the standard deviations are presented in Table 4.

The precision of the analytical procedure was checked by comparing  $TV$  with  $F = 3.89$ . For each recovery function,

since  $TV < F$ , then the deviation of the standard procedure and the residual standard of the recovery function were not significantly different. This analysis validates the use of the recovery function to calculate the real cell number from the measured or experimental values obtained using the MCH-qPCR procedure. When the recovery of DNA from *Salmonella* cells added to the MCH-qPCR is not 100%, application of the recovery function can be used to derive the actual cell number. The detection limit using MCH-qPCR for undigested samples was 30 cells with buffer 1, approximately five cells with buffer 2, and for qPCR alone fewer than five cells. It should be noted that a sample containing lower numbers of *Salmonella* may lead to a false negative result. In this case an enrichment culture or other steps to increase cell concentration would be necessary (Burtscher & Wuertz 2003) before making a confirmatory analysis using MCH-qPCR.

### Application of MCH-qPCR to water samples

While the application of fluorometric qPCR assays (e.g. TaqMan) to environmental samples is an attractive prospect, the difficulty lies in the presence of interfering compounds in the template. Humic acids and fulvic acids have been shown to either autofluoresce or quench fluorescence in such assays (Stults *et al.* 2001), which can lead to overestimation or underestimation of target in the final analysis. Table 2 lists other compounds that can influence the enzymatic amplification of target DNA. Additionally, as was shown above (Table 3), the presence of foreign DNA in the PCR reaction can negatively affect quantitative detection. The methodology proposed herein,

**Table 4** | Parameters related to standard and recovery function

Recovery function	Parameters						Equations used
	$a$	$b$	$r^2$	$S_y$	$S_{x0}$	$TV$	
Calibration curve	-0.139	0.996	0.991	0.145	0.145	--	2–4
Buffer 1, non-digested	-1.035	0.867	0.9786	0.202		1.0156	1, 5, 6
Buffer 2, digested	-1.183	1.205	0.9912	0.124		0.7339	1, 5, 6
Buffer 2, non-digested	-0.964	1.042	0.9559	0.245		2.857	1, 5, 6

**Table 5** | Application of MCH-qPCR to digested water sample

Sample*	qPCR	MCH-qPCR	% Recovery
Control - dH <sub>2</sub> O	1,025 ± 113	496 ± 23	48
River water	573 ± 31	477 ± 86	47

\*Spiked with 1,000 *Salmonella* cells

MCH-PCR and MCH-qPCR, is an effective means of separating these substances and others from nucleic acids ensuring more accurate and reproducible results. The newly developed method was tested on a spiked water sample that was concentrated 100-fold in order to ensure high levels of inhibitors and contaminants. The inhibitory effects of this matrix on amplification were studied by comparing the effectiveness of qPCR and MCH-qPCR using digested nucleic acid as spiked template (Table 5).

Approximately 1,000 cells were added to each reaction. The water control demonstrates the optimal detection scenario for both qPCR and MCH-qPCR. The recovery of *Salmonella* DNA using MCH-PCR for both the control and river samples was nearly identical, and close to the optimal recovery of 50% when employing the correct buffers and enzymatic digestion. Therefore, the beads removed DNA from inhibitory constituents in the original river water and detection by MCH-qPCR was not affected. However, with qPCR, the cell number for the river sample was approximately half of the expected value compared with the control; a reduction in detection that may be attributed to the presence of inhibitory compounds in the digested nucleic acid template. Even when detection by qPCR is possible, the actual cell number in the sample remains unknown since the effect of inhibitors is not quantified. Conversely, MCH-qPCR combined with the recovery function provides the tools to both remove inhibitory compounds from nucleic acids and calculate actual sample cell concentrations.

## CONCLUSIONS

Quantitative measurements made possible by real-time PCR are a valuable tool when assessing the effects that various changes in hybridization conditions have on magnetic

capture hybridization (MCH). The addition of the beads in the qPCR reaction introduces some of the difficulties associated with manipulation of heterogeneous systems. However, these are largely offset by the advantage that beads offer in terms of reducing the detection limit by removal of PCR inhibitors and non-target nucleic acid. Further work must address the optimization of template recoveries using MCH.

The procedures outlined herein, MCH-PCR and MCH-qPCR, have been demonstrated to be useful for the detection of *Salmonella* in water samples containing high levels of PCR inhibitors. The effects of PCR inhibition can be mitigated by the use of beads, and actual sample cell concentrations can be determined by applying a recovery function. The proper choices of buffers for washing and hybridization, and the enzymatic digestion of target DNA prior to hybridization, were found to dramatically improve the capture and quantitative detection of specific nucleic acid.

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## REFERENCES

- Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R. & Swaminathan, B. 2000 *Salmonella* nomenclature. Guest commentary. *J. Clin. Microbiol.* **38**, 2465–2467.
- Burtscher, C. & Wuertz, S. 2003 Evaluation of the use of PCR and reverse transcriptase PCR for detection of pathogenic bacteria in biosolids from anaerobic digestors and aerobic composters. *Appl. Environ. Microbiol.* **69**, 4618–4627.
- Chandler, D. P., Cebula, S. J., Schuck, B. L., Weaver, D. W., Anderson, K. K., Egholm, M. & Brockman, F. J. 2000 Affinity purification of DNA and RNA from environmental samples with peptide nucleic acid clamps. *Appl. Environ. Microbiol.* **66**, 3438–3445.
- Chen, J. R. & Griffiths, M. 1998 Detection of verotoxigenic *Escherichia coli* by magnetic capture hybridization PCR. *Appl. Environ. Microbiol.* **64**, 147–152.



- Chiu, C. H. & Ou, J. T. 1996 Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. *J. Clin. Microbiol.* **34**, 2619–2622.
- Cullen, D. W. & Hirsch, P. R. 1998 Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biol. Biochem.* **30**, 983–993.
- Funk, W., Dammann, V. & Donnevert, G. 1995 *Quality Assurance in Analytical Chemistry*. VCH Publishers, New York.
- Harry, M., Gambier, B., Bourezgui, Y. & Garnier-Sillam, E. 1999 Evaluation of purification procedures for DNA extracted from organic rich samples: Interference with humic substances. *Analysis* **27**, 439–442.
- Hoorfar, J., Ahrens, P. & Rådström, P. 2000 Automated 5' nuclease PCR assay for identification of *Salmonella enterica*. *J. Clin. Microbiol.* **38**, 3429–3435.
- Jacobsen, C. S. 1995 Microscale detection of specific bacterial DNA in soil with a magnetic capture-hybridization and PCR amplification assay. *Appl. Environ. Microbiol.* **61**, 3347–3352.
- Langrell, S. R. H. & Barbara, D. J. 2001 Magnetic capture hybridisation for improved PCR detection of *Nectria galligena* from lignified apple extracts. *Plant Mol. Biol. Rep.* **19**, 5–11.
- Loge, F. J., Thompson, D. E. & Call, D. R. 2002 PCR detection of specific pathogens in water: a risk-based analysis. *Environ. Sci. Technol.* **36**, 2754–2759.
- Maher, N., Dillon, H. K., Vermund, S. H. & Unnasch, T. R. 2001 Magnetic bead capture eliminates PCR inhibitors in samples collected from the airborne environment, permitting detection of *Pneumocystis carinii* DNA. *Appl. Environ. Microbiol.* **67**, 449–452.
- Mangiapan, G., Vorkuka, M., Shouls, L., Cadranel, J., Lecossier, D., vanEmbden, J. & Hance, A. J. 1996 Sequence capture-PCR improves detection of mycobacterial DNA in clinical specimens. *J. Clin. Microbiol.* **34**, 1209–1215.
- Marsh, I., Whittington, R. & Millar, D. 2000 Quality control and optimized procedure of hybridization capture-PCR for the identification of *Mycobacterium avium* subsp *paratuberculosis* in faeces. *Mol. Cell. Probes* **14**, 219–232.
- Miller, D. N., Bryant, J. E., Madsen, E. L. & Ghiorse, W. C. 1999 Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* **65**, 4715–4724.
- Shapir, N., Goux, S., Mandelbaum, R. T. & Pussemier, L. 2000 The potential of soil microorganisms to mineralize atrazine as predicted by MCH-PCR followed by nested PCR. *Can. J. Microbiol.* **46**, 425–432.
- Straub, T. M. & Chandler, D. P. 2003 Towards a unified system for detecting waterborne pathogens. *J. Microbiol. Methods* **53**, 185–197.
- Stults, J. R., Snoeyenbos-West, O., Methe, B., Lovley, D. R. & Chandler, D. P. 2001 Application of the 5' fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. *Appl. Environ. Microbiol.* **67**, 2781–2789.
- Tebbe, C. C. & Vahjen, W. 1993 Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl. Environ. Microbiol.* **59**, 2657–2665.
- Wilson, I. G. 1997 Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.* **63**, 3741–3751.

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