Development of an *Escherichia coli* K12-specific quantitative polymerase chain reaction assay and DNA isolation suited to biofilms associated with iron drinking water pipe corrosion products

Jingrang Lu, Tammie L. Gerke, Helen Y. Buse and Nicholas J. Ashbolt

ABSTRACT

A quantitative polymerase chain reaction assay (115 bp amplicon) specific to *Escherichia coli* K12 with an ABI[TM] internal control was developed based on sequence data encoding the *rfb* gene cluster. Assay specificity was evaluated using three *E. coli* K12 strains (ATCC W3110, MG1655 & DH1), 24 non-K12 *E. coli* and 23 bacterial genera. The biofilm detection limit was 10^3 colony-forming units (CFU) *E. coli* K12 mL^{-1}, but required a modified protocol, which included a bio-blocker *Pseudomonas aeruginosa* with ethylenediaminetetraacetic acid buffered to pH 5 prior to cell lysis/DNA extraction. The novel protocol yielded the same sensitivity for drinking water biofilms associated with Fe_3O_4 (magnetite)-coated SiO_2 (quartz) grains and biofilm-surface iron corrosion products from a drinking water distribution system. The novel DNA extraction protocol and specific *E. coli* K12 assay are sensitive and robust enough for detection and quantification within iron drinking water pipe biofilms, and are particularly well suited for studying enteric bacterial interactions within biofilms.

Key words | drinking water biofilm, *Escherichia coli* K12, iron oxide/oxyhydroxide, qPCR assay

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INTRODUCTION

Biofilms in drinking water distribution systems (DWDSs) are associated with corrosion products and may act as reservoirs for fecal indicators and enteric pathogens that may be in those systems (van der Wende *et al.* 1989; Ashbolt 2003; Berg 2008; Yang *et al.* 2011). While biofilms sequester pathogens, the subsequent release of concentrated pathogenic microbes from biofilms into the main flow could adversely impact the health of drinking water consumers (Storey & Ashbolt 2003).

When undertaking a quantitative microbial risk assessment of drinking water pathogens, densities are generally too low to be assayed directly, hence *Escherichia coli* or other indicator bacteria are used to estimate the level of potential fecal contamination (Medema *et al.* 2006). Furthermore, given the possibility of active but non-culturable *E. coli* in distribution system biofilms (Juhna *et al.* 2007), it is desirable to use a quantitative molecular method, such

as quantitative polymerase chain reaction (qPCR), to assay *E. coli* in biofilms of full-scale and pilot-scale DWDSs.

E. coli K12 strains are classified as nonpathogenic because they do not colonize the human gut (Smith 1975), are devoid of all known *E. coli* virulence genes (Mühldorfer & Hacker 1994) and have never been reported to cause any disease (Kuhnert *et al.* 1995). However, because of the ease of genetic manipulation, a variety of *E. coli* K12 recombinant DNA strains exist, making identification difficult using culture or currently available molecular methods.

E. coli K12 is an ideal microorganism to use for examining the behavior of enteric bacterial pathogens in a pipe loop or biofilm reactor system designed to mimic a DWDS. Previous studies have shown that some *E. coli* K12 derivative strains contain a mutation within the *rfb* gene cluster (*rfb-50*) that results in the lack of certain virulence genes (O antigens) (Liu

& Reeves 1994). Also, two conventional PCR assays have been developed based on the *rfb* gene cluster DNA sequences to distinguish the AB311 side lineage of *E. coli* K12 (Kuhnert *et al.* 1995). Because of mutated regions within the *rfb* gene cluster, the genetic characteristics of *E. coli* K12 strains allow for the development of a specific assay either for a group of *E. coli* K12 strains or a single strain. However, to date, no qPCR assay specific to *E. coli* K12 has been reported.

Unlike many other environmental samples, drinking water biofilms collected from iron drinking water pipes or bioreactors generally contain particulates of iron pipe corrosion products and mineral phases precipitated from the drinking water, which make DNA isolation difficult and/or result in PCR inhibition (Kreader 1996; Teng *et al.* 2008). In previous experiments, iron phase PCR inhibition was reduced by the addition of bovine serum albumin, T4 Gene 32 Protein (Kreader 1996) or ethylenediaminetetraacetic acid (EDTA) (Teng *et al.* 2008). However, these methods did not attempt to minimize DNA sorption by iron phases during the extraction of biofilm DNA, which we consider necessary to improve the sensitivity for molecular identification of microorganisms from iron-enriched biofilms.

The aim of this study was to develop an *E. coli* K12 specific qPCR assay and to optimize DNA extraction for use in the study of iron-enriched biofilms, so as to identify spiked *E. coli* K12 against the possible background of other *E. coli*. Also, as *E. coli* is the most commonly used fecal indicator, *E. coli* K12 provides an excellent analog to target enteric bacteria that may contaminate treated drinking water and be sequestered within distribution system biofilms.

MATERIALS AND METHODS

Bacterial strains and biofilm materials

Bacterial strains and culture

The bacterial strains used in this study (Table 1) were stored at $-80\,°C$. Strains were grown on Luria–Bertani Broth (Invitrogen, Carlsbad, CA) overnight, pelleted by centrifugation and DNA extracted as described below or whole cells enumerated and used to spike samples.

Model drinking water distribution system biofilms grown on non- and Fe₃O₄-coated SiO₂ grains

Perforated 316 stainless steel sheeting was rolled and welded to create cylindrical baskets that were filled with approximately 50 g of either non- or Fe_3O_4-coated SiO_2 grains, approximately 3 mm in diameter. Sterile baskets were inserted and maintained for four months in a side stream receiving drinking water off a pipe loop model DWDS (clear schedule 80 unplasticized polyvinyl chloride (PVC), 16.5 m long by 15.2 cm ID containing 264 L). The loop was wrapped with black plastic to minimize light entry and filled with municipal drinking water that was disinfected with utility-supplied free chlorine (mean concentration of $0.71\,mg\,L^{-1}$). The flow rate through each basket was $100\,mL\,min^{-1}$. Water quality was maintained by draining approximately 2.5 to $4\,L\,min^{-1}$ from the pipe loop and allowing entry of fresh drinking water containing an appropriate disinfectant residual. Baskets were collected after four months, and biofilms were harvested via probe sonication ($2 \times 30\,s$) (ultrasonic homogenizer, Braun Biotech International, Melsungen, Germany) in 10 mL of autoclaved drinking water of approximately pH 8.5 that had been coarse-filtered (Miracloth of 22–25 μm pore size (Calbiochem, San Diego, CA, USA)), hereafter referred to as CFTW (coarse-filtered tap water). The resulting biofilm homogenates obtained from uncoated SiO_2 grains are referred to as low-iron biofilms, while those from Fe_3O_4-coated SiO_2 grains are described as iron-enriched biofilms.

Biofilms obtained from unlined cast iron drinking water pipe corrosion products

Sample scrapings of the surface layer of iron corrosion products/biofilms (100 mg) were obtained from a freshly exhumed (within 5 h) cast iron drinking water pipe. The pipes were kept moist until processing of biofilm/corrosion product in CFTW as described above, and are also referred to as iron-enriched biofilms.

Protocol 1 for DNA extraction and purification for low-iron biofilms

Biofilm homogenates (1 mL) were centrifuged at 15,000 **g** for 8 min, and the resulting biomass was disrupted and lysed

Table 1 | Bacterial strains used to check the specificity of the qPCR assay for *E. coli* K12

Strains	Source	Strains	Source	Strains	Source
Acinetobacter baumannii	ATCC 19606	*Micrococcus luteus*	ATCC 10240	*E. coli*	EPA isolate 69
Aeromonas hydrophila	ATCC 7966T	*Proteus mirabilis*	ATCC 12453	*E. coli* O157:H7	CDC isolate 302292
Aeromonas hydrophila	ATCC 7966	*Proteus vulgaris*	ATCC 29905	*E. coli* O157:H7	Dairy isolate #1
Aeromonas cavae	ATCC 15468	*Pseudomonas aeruginosa*	ATCC 10145	*E. coli* O157:H7	Dairy isolate #5
Bacillus cereus	ATCC 10876	*Salmonella enteriditis*	ATCC 13076	*E. coli* O157:H7	Dairy isolate #6
Burkholderia cepacia	ATCC 25416	*Serratia marcescens*	ATCC 14756	*E. coli* O157:H7	TX N6021-1, Btype2113
Burkholderia cepacia	ATCC 25416	*Serratia marcescens*	ATCC 13880	*E. coli* O157:H7	TX N6114-2, Btype2521
Campylobacter jejuni	ATCC 29428	*Shewanella putrefaciens*	ATCC 49138	*E. coli* O157:H7	WI N009-6-1, Btype
Citrobacter freundii	ATCC 4391	*Shigella sonnei*	ATCC 25931	*E. coli* O157:H7	ATCC 43889
Citrobacter freundii	ATCC 8090	*Shigella sonnei*	ATCC 9290	*E. coli* O157:H7	ATCC 35150
Enterobacter aerogenes	ATCC 13048	*Staphylococcus aureus*	ATCC 25923	*E. coli* O26	ATCC 12795
Enterobacter aerogenes	ATCC 13048	*Staphylococcus aureus*	ATCC 25923	*E. coli* O111	ATCC 35327
Enterobacter cloacae	ATCC 13047	*Streptococcus pyogenes*	ATCC 19615	*E. coli*	ATCC 51813
Enterococcus faecalis	ATCC 29302	*Yersinia enterocolitica*	ATCC 23715	*E. coli*	ATCC 25922
Enterococcus faecalis	ATCC 19433	*Escherichia coli*	EPA isolate 8	*E. coli*	ATCC 11229
Enterococcus faecium	ATCC 19434	*E. coli*	EPA isolate AD#1	*E. coli*	ATCC 43651
Lactobacillus acidophilus	ATCC 314	*E. coli*	EPA isolate 58	*E. coli*	ATCC 10407
Klebsiella oxytoca	ATCC 13182	*E. coli*	EPA isolate 59	[a]*E. coli* K12, DH1	ATCC 33849
Klebsiella pneumoniae	ATCC 13882	*E. coli*	EPA isolate 62	[a]*E. coli* K12, MG1655	ATCC 700926
Klebsiella pneumoniae	ATCC 31488	*E. coli*	Ohio river isolate R-6	[a]*E. coli* K12 W3110	ATCC 27325
Listeria monocytogenes	Scott O2	*E. coli*	EPA isolate 66		

[a]Test for K12 specific assay: positives shown with gray shading.

using a Mini-Beadbeater-16 (BioSpec Products, Inc., Bartlesville, OK) in 300 μL $1 \times$ T&C (cell and tissue) solution for 2×30 s and DNA extracted using the MasterPure Complete DNA Purification Kit™ (Epicentre Biotechnologies, Madison, WI). Triplicate samples were centrifuged at 10,000 g for 8 min, supernatant transferred to sterile tubes, 1 μL of proteinase K (20 mg mL^{-1}) added and the proteins digested on a heating block at 65 °C for 15 min while shaking at 1,000 rpm. The lysate supernatants were placed on ice for 30 s and then an equal volume of protein precipitation solution (MPC, Epicentre Biotechnologies, Madison, WI) was added for remaining protein precipitation. The protein precipitate was removed by centrifugation at 10,000 *g* for 8 min. Purification and concentration of the genomic DNA from the supernatant was accomplished using Genomic DNA Clean & Concentrator™ as recommended by the manufacturer (Zymo Research Corporation, Irvine, CA). The resulting DNA was eluted with 100 μL molecular biology grade water (Thermo Fisher Scientific, Madison, WI) and DNA concentrations estimated with a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, Delaware).

Protocol 2 for DNA extraction and purification for iron-enriched biofilm samples

Protocol 2 was developed based on cofactors affecting release of DNA from iron-enriched materials, as *E. coli* K12 spiked into a mixture of CFTW with 25 mg of fine-grained Fe_3O_4

(magnetite; GFS Chemicals Inc., Columbus, OH) provided unamplifiable DNA using Protocol 1. To modify Protocol 1, a series of experiments were designed with positive controls (*E. coli* K12 and *E. coli* K12 plus cofactors), negative controls (water and water plus cofactors) and various densities (10–10^7) of *E. coli* K12 plus cofactors. All tests with Protocol 2 included Fe_3O_4 (25 mg magnetite, GFS Chemicals Inc., Columbus, OH) with the following combinations of cofactors: EDTA (0, 10, 50, 100, 220 & 500 mM, Sigma-Adrich); 100 mM sodium acetate buffer prepared from sodium acetate/acetic acid (Sigma-Aldrich Corp., St Louis, MO) and adjusted to pH 5.0, 5.8 or left at the unadjusted CFTW pH of 8.5; and a bio-blocking agent (*Pseudomonas aeruginosa*, 10^7 or 10^8 colony-forming units (CFU) mL^{-1}). The treatment of biofilm homogenates using Protocol 2 was identical to Protocol 1 except that samples were resuspended with the *P. aeruginosa* bio-blocking agent, EDTA or both, with the pH adjusted and held at room temperature with brief vortexing every 10 min for a total of 30 min, before lysis/DNA extraction as described for Protocol 1. Positive control samples were mixtures of *E. coli* K12 and CFTW, and *E. coli* K12, CFTW with cofactors. Negative controls were only CFTW, and CFTW with cofactors.

Quantitative PCR

Primer design

Primers K12F1 (5′-TAAAGTAACCTTGATCGAAG-3′), K12R1 (5′-ATTCCTAAAGAAAGTATCTATTC-3′), and the TaqMan probe K12prob1 (5′-(6-FAM) AACGTACCAGCA-TAAATGATCCT (TAMRA)-3′) (ABI) were designed based on the alignment of *rfb* gene cluster partial sequences using Primer Express software (Applied Biosystems). Alignment of all available DNA sequences was conducted using Sequencher® version 4.9 (Gene Codes Corporation, Ann Arbor, MI). The 21 sequences (eight *E. coli* K12 strains and other *E. coli* strains) were downloaded from GenBank, including *E. coli* K12 strains DH1, MG1655 and W3110 (Table 1). The primers were selected to amplify a 115 bp region of the *rfb*-50 mutation described by Liu & Reeves (1994).

 The sequence specificity of the *E. coli* K12 assay was confirmed using Basic Local Alignment Search Tool (BLAST) to search in the European Molecular Biology Laboratory

(EMBL) and GenBank databases for published sequences identical to the primers K12F1, K12R1 and K12prob1. At the date of evaluation (August 14, 2012), all primers and the probe were 100% identical to all nine *E. coli* K12 sequences (eight strains), but the K12F1 primer also matched an extra non-*E. coli* sequence, *Corynebacterium jeikeium* with 100% identity. The primers and qPCR conditions were tested and optimized by running annealing temperature gradients and using serial dilutions (10–10^7) *E. coli* K12 DNA templates. Primers were tested for host specificity against DNA extracted from the bacteria listed in Table 1. The qPCR assay was performed on a 7900 HT Fast Real-Time Sequence Detector (Applied Biosystems). Reaction mixtures (20 μL) contained 10 μL 2× qPCR Master Mix (Applied Biosystems), 0.2 μM primers, 0.08 μM probe (final concentration) and 2 μL of template DNA. Initial DNA treatment consisted of 50 °C for 2 min with UNG (uracil-*N*-glycosylase) to prevent carryover contamination, then 95 °C for 10 min for DNA denaturing. The following quantification cycling protocol was used: 40 cycles at 95 °C for 15 s and 56 °C for 30 s with an extension at 72 °C for 30 s and a final hold at 72 °C for 5 min, with qPCR reactions for each DNA sample undertaken in quadruplicate.

Standard curves, detection limit and amplification efficiency of biofilm DNA extractions

Standard curves were constructed with 10-fold serial dilutions of *E. coli* K12 strain ATCC 33849 (DH1) mixed with biofilms, which were *E. coli* K12-free biofilm homogenate in the non-spiking control biofilm samples, harvested from non- and Fe_3O_4-coated quartz grains, using Protocols 1 and 2, respectively. Each standard curve was prepared in quadruplicate and ranged from 10 to 10^7 CFU (assayed using Luria–Bertani medium with 1.5% agar). Dilutions were freshly prepared for each experiment from one *E. coli* K12 (DH1) aliquot stored at −20 °C. To assay for qPCR inhibitors each DNA extract was assayed neat, after a 10-fold dilution and with the addition of the TaqMan Exogenous Internal Positive Control Reagents (a VIC-labeled probe) manufactured by ABI™. Furthermore, each qPCR run included a DNA standard curve in the first row of the 96-well plate, and a no-template control for each row of each 95-well plate assayed. The qPCR efficiency

was calculated using the following equation: Efficiency % = $100 \times (10^{(-1/\text{slope})} - 1)$ (Applied Biosystems).

Data analysis

Experiments designed for pairwise comparison were statistically analyzed by analysis of variance (ANOVA) using SAS Systems version 9.2 (SAS, Cary, NC).
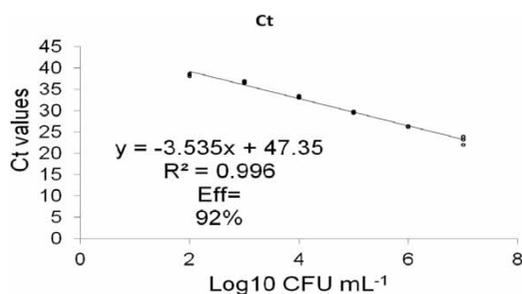
RESULTS

E. coli K12 qPCR specificity and sensitivity using Protocol 1

The specificity of the *E. coli* K12 qPCR assay was first demonstrated with DNA isolated using Protocol 1 from overnight cultures of three *E. coli* K12 stains (W3110, MG1655 and DH1), 24 *E. coli* non-K12 water isolates and 35 other bacterial strains representing 23 genera (Table 1).

The analytical sensitivity (detection limit) of the novel qPCR assay was assessed using DNA extracted from *E. coli* K12 directly spiked into 1 mL of CFTW (positive control), and was approximately 10^3 cells mL^{-1} or 20 cell reaction^{-1} using DNA isolated with Protocol 1. The efficiency of qPCR amplification was 92% (Figure 1).

However, in the presence of Fe$_3$O$_4$ (25 mg mL^{-1}) no *E. coli* K12 amplification was detected even when the starting *E. coli* concentration was 10^5 CFU mL^{-1} and included 10-fold dilutions of the template, whereas the internal control indicated no PCR inhibition (Table S1, available online at http://www.iwaponline.com/wh/012/203.pdf). Based on these results Protocol 2 was developed to improve DNA recovery from iron-enriched biofilms.

Figure 1 | A standard curve of *E. coli* K12 specific assay for DNA extracted in triplicate using Protocol 1.

Development of Protocol 2

The addition of 10^7 CFU mL^{-1} of *P. aeruginosa* to *E. coli*-CFTW-Fe$_3$O$_4$ mixtures resulted in improved detection of the *E. coli* qPCR probe signals (Ct values decreased), compared to the previous test where no signal was detected when *P. aeruginosa* was absent from the mixture. Nonetheless, the results were still approximately 4-log units less than the positive non-Fe$_3$O$_4$ controls (Table 2). In an attempt to improve the *E. coli* qPCR probe signals, *P. aeruginosa* was increased 10-fold; from 10^7 to 10^8 CFU mL^{-1}, which resulted in a 3-log increase in the qPCR sensitivity. This improved sensitivity was also evident for *E. coli* K12 at 10^4 to 10^5 CFU mL^{-1} (Table 3).

To further improve *E. coli* qPCR sensitivity, three different concentrations of EDTA (10, 50 and 100 mM) were added to the *E. coli*-Fe$_3$O$_4$-CFTW-*P. aeruginosa* mixture. However, working with *E. coli* at 10^6 CFU mL^{-1} and *P. aeruginosa* at 10^8 CFU mL^{-1}, regardless of the EDTA concentration there was no significant improvement in qPCR Ct values (Table S2, available online at http://www.iwaponline.com/wh/012/203.pdf) at the CFTW pH of 8.5. To facilitate the benefit of EDTA addition and to reduce DNA sorption to iron phases the final pH was adjusted to 5.0 or 5.8, using the acetate buffer (100 mM) prior to cell lysis-DNA extract, which significantly improved the qPCR detection of *E. coli* K12 at 10^4 and 10^5 CFU mL^{-1} (Table 4). To further assess the optimal concentration of EDTA at pH 5.0, Ct values were assessed from 55 to 220 mM EDTA (Table S3, available online at http://www.iwaponline.com/wh/012/203.pdf).

Table 2 | Impact of *P. aeruginosa* blocking agent on *E. coli* K12 qPCR detection (Ct) with Fe$_3$O$_4$ present[a]

Sample	*E. coli* K12 (10^5 CFU mL^{-1})	*P. aeruginosa* (10^8 CFU mL^{-1})	Mean Ct	STDEV Ct
Positive control	+	−	22.5	0.42
Negative 1	−	−	BD[b]	
Negative 2	−	+	BD	
No blocking	+	−	BD	
With blocking	+	+	26.7	0.19

[a]Each sample contained 1 mL of CFTW (pH 8.5), 25 mg of Fe$_3$O$_4$ except positive control, all with no pH adjustment. The mean cycle times (Ct) to reach detection by qPCR are based on four replicates (i.e. the lower the Ct the more sensitive the detection limit; each 2.5 Ct equates to about 1 log$_{10}$ of *E. coli*).
[b]BD – below detection.

Table 3 | Impact of *P. aeruginosa* density on *E. coli* K12 qPCR detection with Fe_3O_4 present ±EDTA (110 mM)[a]

Sample	*P. aeruginosa* (CFU mL^{-1})	Mean Ct	STDEV Ct	Comparisons of Ct between two densities of *P. aeruginosa*	P (*t*-test)
No EDTA 1	10^7	BD[b]		1 vs 2	NA[c]
No EDTA 2	10^8	37.0	0.03		
EDTA 1	10^7	36.1	0.05	1 vs 2	< 0.0001
EDTA 2	10^8	32.9	0.43		

[a]Each sample contained 1 mL of CFTW (pH 8.5), 25 mg of Fe_3O_4 and 10^4 CFU mL^{-1} of *E. coli* K12, all with no pH adjustment. The mean cycle times (Ct) to reach detection by qPCR are based on four replicates .
[b]BD – below detection.
[c]NA – not available.

Table 4 | Impact of pH on *E. coli* K12 qPCR detection with Fe_3O_4 present[a]

Sample number	*E. coli* K12 (CFU mL^{-1})	pH	Mean Ct	STDEV Ct	Comparisons of Ct between control and each acetate buffered pH	P (*t*-test)
0[b]	10^4	8.5	36.0	0.54		
1[c]	10^4	4.5	35.6	0.17	0 vs 1	0.562
2[c]	10^4	5.0	34.7	0.26	0 vs 2	0.052
3[c]	10^4	5.8	34.1	0.35	0 vs 3	0.014
0[b]	10^5	8.5	34.0	0.02		
1[c]	10^5	4.5	32.4	0.21	0 vs 1	0.023
2[c]	10^5	5.0	30.6	0.37	0 vs 2	0.002
3[c]	10^5	5.8	30.4	0.55	0 vs 3	0.001

[a]Each sample contained 1 mL of CFTW, 25 mg of Fe_3O_4, 110 mM of EDTA, 10^8 CFU mL^{-1} of *P. aeruginosa* and 10^4 or 10^5 CFU mL^{-1} of *E. coli* K12. The mean Ct values are based on four replicates.
[b]Positive control (CFTW) no buffer, pH 8.5.
[c]Acetate buffered EDTA (final pH 4.5–5.8).

Using a two-way ANOVA the ideal combination for maximum DNA extraction and recovery for *E. coli* K12 in the presence of iron phases consisted of 10^8 CFU mL^{-1} of *P. aeruginosa* bio-blocking agent and EDTA at 110 mM buffered to pH 5.0. This combination significantly increased the detection of *E. coli* K12 qPCR signal (Table 5) and the detection limit was close to the positive control of approximately 10^3 CFU mL^{-1}.

Evaluation of Protocols 1 and 2 for *E. coli* K12 assay in low-iron and iron-enriched biofilms

No significant difference was detected in the DNA extractions from *E. coli* K12 added to 4-month-old biofilms grown in a drinking water pipe-loop on either non- or Fe_3O_4-coated SiO_2 grains compared to the positive controls (Table 6). Furthermore, the *E. coli* K12 qPCR detection limited for the iron-enriched biofilms was 50 CFU per reaction using Protocol 2. Overall, Protocol 2 provided superior recovery of *E. coli* K12 compared to Protocol 1 for the positive control, iron-enriched biofilms (P [*t*-test] <0.0001, Figure 2).

Quality control tests of the *E. coli* K12 assay in the above matrices conducted using the internal positive control (IPC) indicated there was no qPCR inhibition for spiked *E. coli* K12 when using Protocol 2 to recover DNA from iron-enriched biofilms (Table S4, available online at http://www.iwaponline.com/wh/012/203.pdf).

DISCUSSION

There were two major challenges in developing a qPCR assay to resolve *E. coli* K12 strains from other *E. coli* within drinking water pipe biofilms: (1) limited nucleotide region available for selecting unique qPCR primers and probes; and (2) DNA recovery from iron-enriched biofilms. Kuhnert *et al*. (1995) developed an end-point PCR assay with primers based on the O-antigen gene cluster (*rfb*) of *E. coli* K12 to differentiate *E. coli* K12-derived strains from other *E. coli* strains used in the laboratory or isolated from human and other environments. Subsequent *E. coli* phylogenetic and evolution research undertaken by Sims & Kim (2011) used a newly developed approach, the feature frequency profile (FFP) method based on a whole-genome phylogeny of the *Escherichia coli/Shigella* group. They were able to determine two *E. coli* K12 wild strains (W3110 and MG1655) and a derivative, strain DH1 within phylogroup A, which includes gastrointestinal tract

Table 5 | Combination effect of bio-blocking P. aeruginosa, pH, Fe$_3$O$_4$ and EDTA on E. coli K12 qPCR detection[a]

Sample#	E. coli K12 (CFU mL^{-1})	P. aeruginosa (CFU mL^{-1})	pH[b]	EDTA (mM)	Mean Ct	STDEV Ct	Ct between control (0) and treatment combination	P (t-test)
0	10^3	0	8.5	0	37.5	0.33		
1	10^3	10^8	5.0	110	36.8	0.31	0 vs 1	0.698
2	10^3	0	8.0	110	BD[c]		0 vs 2	NA[d]
3	10^3	0	5.0	110	BD		0 vs 3	NA
0	10^4	0	8.5	0	34.0	0.23		
1	10^4	10^8	5.0	110	33.8	0.19	0 vs 1	0.156
2	10^4	0	8.0	110	BD		0 vs 2	NA
3	10^4	0	5.0	110	36.8	0.50	0 vs 3	< 0.001
0	10^5	0	8.5	0	28.6	0.48		
1	10^5	10^8	5.0	110	29.9	0.14	0 vs 1	0.109
2	10^5	0	8.0	110	BD		0 vs 2	NA
3	10^5	0	5.0	110	33.3	0.28	0 vs 3	< 0.0001

[a]Each sample contained 1 mL of CFTW and except for positive controls (sample 0), 25 mg of Fe$_3$O$_4$ and 110 mM of EDTA. The mean Ct values are based on four replicates.
[b]Acetate buffered EDTA (final pH 5.0 or 8.0).
[c]BD – below detection.
[d]NA – not available.

Table 6 | Comparisons of E. coli K12 assay qPCR standard curve generated from DNA extracted from control water versus biofilm-only, Fe$_3$O$_4$-only or biofilm formed on Fe$_3$O$_4$[a]

	Positive control CFTW	Biofilm-only	Fe$_3$O$_4$-only	Biofilm formed on Fe$_3$O$_4$
Linear equation	$-3.533x + 47.36$	$-3.535x + 47.35$	$-3.576x + 46.63$	$-3.566x + 46.74$
R^2	0.996	0.996	0.997	0.997
qPCR efficiency[b]	92%	92%	90%	91%
P-values (t-test, two tailed) Control vs		1.000	0.892	0.934

[a]E. coli K12 standard curve in 1 mL of CFTW ranged 10^1–10^7 CFU mL^{-1} with four replicates for the LOG$_{10}$(densities) examined, total observation $N = 20$ (actual analyzed data) for each experiment.
[b]Efficiency was $100 \times (10^{(-1/slope)} - 1)$.
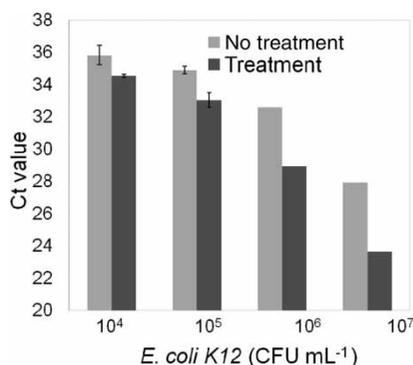
Figure 2 | Comparison of qPCR Ct values for DNA extracted by Protocols 1 versus 2 targeting E. coli K12 spiked into 100 mg of iron-enriched biofilm from cast iron water mains.

commensals that all lack a wide range of virulence factors. Thus it was reasonable to assume that the *rfb* cluster could be used to differentiate E. coli K12 from other environmentally-occurring E. coli strains. The specificity test described in this paper showed that the qPCR assay based on the last gene of the *rfb* cluster is not only specific to differentiate E. coli K12 from other E. coli, but also a wide range of bacterial isolates from river and drinking water environments (Table 1).

The second challenge was the recovery of amplifiable DNA from iron-enriched drinking water biofilm materials. First we suspected metal-based inhibition as reported by Wilson (1997) for iron co-extracted with DNA, which can

inhibit PCR, similarly as reported by Schwartz *et al.* (1998) for biofilms containing copper pipe corrosion products. However, data in this paper demonstrated that the presence of Fe_3O_4 (a representative pipe corrosion product (Gerke *et al.* 2008)) lowered the DNA yield rather than inhibiting PCR. The reduced yield was likely because negatively charged biofilm DNA would be expected to bind to Fe_3O_4 (average point of zero charge ∼pH 6.9), given the net positive surface charge on magnetite in most drinking waters (pHs range typically between 7 and 9). Indeed, the adsorption of DNA to Fe_3O_4 has been used for extraction of genomic DNA (Shi *et al.* 2009).

Nowack & Sigg (1996) showed that the adsorption characteristics of a variety of divalent and trivalent ions to α-FeOOH (goethite) in a system with metal–EDTA complexes were affected by pH. The utility of coupling mineral surface charge and low pH on EDTA-mineral sorption to minimize adsorption of cell-extracted DNA to Fe_3O_4 and possibly other corrosion products was demonstrated in this paper. After a number of combinations (Tables 2–5) the preferred mix of cofactors (final Protocol 2) prior to cell lysis included: 10^8 CFU mL^{-1} of *P. aeruginosa* as a bio-blocking agent, and 110 mM EDTA in a pH 5.0 acetate buffer (100 mM). We utilized *P. aeruginosa* firstly as it is often found in water and simply used a high level to be sure of background DNA to aid in quenching DNA sorption sites, and secondly as it is readily spiked and lysed in a consistent way. Using Protocol 2 for iron-enriched biofilms resulted in no significant PCR inhibition and the *E. coli* K12 qPCR signal sensitivity was increased to that of Protocol 1 (Figure 2) such that the detection limit was close to the positive control of approximately 10^3 CFU mL^{-1} (Table 5). Therefore Protocol 2 is proposed as a reliable approach for obtaining amplifiable DNA from iron-enriched biofilms (Table 6). The detection limit was often higher than 20 cells per reaction in iron-enriched biofilm matrices (similar to those achieved with Protocol 1 for biofilms growing on low-iron biofilms). These detection limits were also similar to previously published results for environmental matrices (Schwartz *et al.* 1998; Kikuchi *et al.* 2002; Anders *et al.* 2009; Hoefel *et al.* 2009), which ranged from 100 to 250 cells per reaction.

The *E. coli* K12 qPCR assay was developed for studying pathogenic and fecal indicator bacteria attachment and biofilm ecology. Previously, culture-based methods have been used in most studies that examined the presence of *E. coli* in biofilms (Wingender & Flemming 2004). However, culture-based methods have the following limitations: time required to visualize growth (days), inaccuracy due to the inability to grow stressed and viable but non-cultural cells (McFeters *et al.* 1986; Juhna *et al.* 2007), antagonistic organism interference, lack of specificity, and inaccuracy because of cell clumping or intertwining with other biofilm components (Rompre *et al.* 2002). Thus, the novel qPCR assay with high specificity and sensitivity combined with the novel DNA extraction protocol (Protocol 2) should allow more accurate assessments of bacterial communities in drinking water biofilms enriched with iron corrosion products. It will also aid in advancing the understanding of the movement of pathogenic microorganisms through DWDSs.

CONCLUSIONS

In this study, an *E. coli* K12-specific qPCR assay was developed based on the sequences encoding the *rfb* gene cluster. This assay was able to amplify both wild type *E. coli* K12 strains and derivatives with a detection limit of approximately 10^3 cell mL^{-1} or approximately 20 cells per reaction. With the addition of a bio-blocker, EDTA, and a pH adjustment of the mixture prior to lysing the sensitivity was significantly increased even in the presence of iron oxides/oxyhydroxides. The novel DNA extraction protocol and *E. coli* K12-specific qPCR assay provides a model fecal indicator to monitor *E. coli* and similar bacterial pathogens in drinking water biofilms.

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