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BIOTECHNOLOGY METHODS

Diagnostics method for the rapid quantitative detection and identification of low-level contamination of high-purity water with pathogenic bacteria

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Abstract High-purity water (HPW) can be contaminated with pathogenic microorganisms, which may result in human infection. Current culture-based techniques for the detection of microorganisms from HPW can be slow and laborious. The aim of this study was to develop a rapid method for the quantitative detection and identification of pathogenic bacteria causing low-level contamination of HPW. A novel internally controlled multiplex real-time PCR diagnostics assay was designed and optimized to specifically detect and identify *Pseudomonas aeruginosa* and the *Burkholderia* genus. Sterile HPW, spiked with a bacterial load ranging from 10 to 10^3 cfu/100 ml, was filtered and the bacterial cells were removed from the filters by sonication. Total genomic DNA was then purified from these bacteria and subjected to testing with the developed novel multiplex real-time PCR diagnostics assay. The specific *P. aeruginosa* and *Burkholderia* genus assays have an analytical sensitivity of 3.5 genome equivalents (GE) and 3.7 GE, respectively. This analysis demonstrated that it was possible to detect a spiked bacterial load of 1.06×10^2 cfu/100 ml for *P. aeruginosa* and 2.66×10^2 cfu/100 ml for *B. cepacia* from a 200-ml filtered HPW sample. The rapid diagnostics method described can reliably detect, identify, and quantify low-level contamination of HPW with *P. aeruginosa* and the *Burkholderia* genus in <4 h. We propose that this rapid diagnostics method could be applied to the pharmaceutical and clinical sectors to assure the safety and quality of HPW, medical devices, and patient-care equipment.

Keywords *Burkholderia* genus · *Pseudomonas aeruginosa* · Spiked high-purity water samples

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Introduction

The microbial monitoring of high-purity water (HPW), medical devices, and patient-care equipment is often limited by lengthy conventional microbial culture-based methodologies for the detection and identification of contaminating microorganisms. In addition to culture-based methodologies, biochemical tests may be required to specifically identify the contaminating microorganism [4]. Combined, these methods are labor-intensive, time-consuming, and can lead to a significant delay in the detection and identification of the microbial contaminant, thereby deferring the onset of any necessary remedial action [52]. It has also been observed that bacteria present in low-nutrient environments such as HPW, medical devices, and patient-care equipment can enter an altered physiological state [9, 55]. While physiologically active, these bacteria are unable to form colonies on culture media and are therefore undetectable by standard microbial cultivation [13].

HPW is a commonly used raw material in the pharmaceutical industry and clinical sectors. Many studies have demonstrated that pathogenic microorganisms can contaminate pharmaceutical raw materials and finished

products [2, 25, 27, 42]. Microbial contamination of 5–260 cfu/100 ml has been observed in purified water [28]. The microbial analysis of HPW and water purification systems has identified a recurring panel of contaminating microorganisms [7, 30], which includes *Pseudomonas aeruginosa* and members of the *Burkholderia* genus [38, 43].

P. aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium. It has the ability to colonize almost all ecological niches, including nutrient-limited environments [20, 44]. Members of the *Burkholderia* genus are also Gram-negative, motile, aerobic, rod-shaped bacteria that are ubiquitous in the environment [12, 33].

Both *P. aeruginosa* and members of the *Burkholderia* genus have also been identified as significant hospital-acquired infectious agents [10, 14, 33]. In the hospital environment, pathogens can be transmitted via person-to-person contact [17, 49]. Other significant routes of infection for these bacteria are through the contamination of hospital environmental reservoirs [8, 32, 53] and the ability of these bacteria to colonize medical devices and patient-care equipment. For example, medical devices and clinical equipment such as endoscopes, bronchoscopes, dialysis tubing, and respiratory devices, have all been shown to act as a source of *P. aeruginosa* and *Burkholderia* species hospital-acquired infections (HAI) [1, 47, 50, 57].

In recent years, significant advances have been made in nucleic acid diagnostics (NAD) technologies for the simultaneous detection and identification of specific microorganisms [36]. Due to shorter turnaround time to results, automation, the high specificity and low limits of detection achieved, NAD technologies are now being routinely used in the clinical, environmental, and food industry sectors [6, 18, 34].

In this study, we report the development of a novel multiplex real-time PCR diagnostics assay for the rapid, quantitative detection and identification of low-level contamination of HPW with *P. aeruginosa* and the *Burkholderia* genus.

Materials and methods

Bacterial strains, culture media, and growth conditions

Twenty-six *Pseudomonas* species/strains and ten *Burkholderia* species were used in this study (Supplementary Table S1). Fourteen *P. aeruginosa* strains, one *P. putida*, and six *Burkholderia* species were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ. Braunschweig, Germany). The remaining *Pseudomonas* and *Burkholderia* species were purchased from BCCM Belgian Co-ordinated Collections of Microorganisms bacterial collection (LMG) (Gent, Belgium).

All *Pseudomonas* and *Burkholderia* species and strains were grown in tryptone soya broth (Oxoid, Cambridge, UK) or R2A broth (Lab M limited, Lancashire, UK) at either 30 or 37 °C for approximately 12 h, or until sufficient growth was observed. For all other microorganisms tested in the study, total genomic DNA was provided from stocks held within this laboratory (Supplementary Table S2).

DNA isolation and quantification

Total genomic DNA was isolated from 1.5 ml of overnight broth culture using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Briefly, 1.5 ml of culture was centrifuged in a bench-top centrifuge at 18,000 × *g* for 2 min. The supernatant was discarded and the pellet resuspended in 180 µl of Buffer ATL (pretreatment for Gram-negative bacteria recommended by manufacturers). Subsequently, steps 2–8 of the purification of total DNA from animal tissue procedure were followed according to the manufacturer's guidelines.

DNA integrity was assessed on a 1 % agarose gel. Concentrations for all total genomic DNA used in this study were determined using the Quant-iT dsDNA HS Assay Kit and the Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). All DNA samples were stored at −20 °C prior to use.

Diagnostics target identification

The diagnostics target genes used in this study were identified by in silico evaluation of a number of genes. Nucleotide sequence information was retrieved from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov), the tmRNA website (http://www.indiana.edu/~tmrna/) and the RNaseP database (http://www.mbio.ncsu.edu/rnasep/). Alignments of these nucleotide sequences were carried out using the clustalW2 multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html) for each putative diagnostics target evaluated in this study.

Conventional and real-time PCR primers and Taqman hydrolysis probe design

Following alignments of the nucleotide sequences of putative target genes, oligonucleotide primers and hydrolysis probes were designed according to recommended general guidelines [15, 45]. Nucleotide sequencing primers were designed to have a melting temperature (Tm) of

Table 1 Oligonucleotide primers and probes used in this study

Name	Function	Sequence (5′–3′)
CIAC F	Forward composite primer for IAC generation	TGAGGAAGACTTATTGGCTGATACCCAACTTGGAATG
CIAC R	Reverse composite primer for IAC generation	CGGTACATTGTGGTCTTTAAGTCTTCACCAGAATAAAATTG
Psd F1	Sequencing forward primer	GGATTTGAACCCCCGTCC
Psd R2	Sequencing reverse primer	AGGATTCGACGCCGGT
BF1	Sequencing forward primer	GAGGAAAGTCCGGACTCC
BF2	Sequencing forward primer	GGCAGGGTGATGGCTAA
BR2	Sequencing reverse primer	GATAAGCCGGATTCTGTGC
IAC F	IAC forward primer	TGAGGAAGACTTATTGGCTG
IAC R	IAC reverse primer	CGGTACATTGTGGTCTTTAAG
IAC P	IAC-specific hydrolysis probe	TYE665-TCCTTAGATGGTACCAGTGCCA- IBRQSp
BF1	*Burkholderia* forward primer	GAGGAAAGTCCGGACTCC
BR1	*Burkholderia* reverse primer	TCTTACCGCACCGTTTCA
Burk P	*Burkholderia* genus-specific hydrolysis probe	FAM-ACACGCGGAACAGGGCAA-BHQ1
PF2	*P. aeruginosa* forward primer	GACAGTCGTTTCGGGTTTAC
PR1	*P. aeruginosa* reverse primer	CGACGACAACTACGCTCT
P. aer P	*P. aeruginosa*-specific hydrolysis probe	HEX-GCATCCCCTAGCGACTGCT-BHQ1

between 60 and 62 °C. The primers used for real-time PCR were designed to have a Tm of 58–61 °C, with all probes designed to have a Tm 7–8 °C higher (Table 1).

For the *P. aeruginosa*-specific diagnostics assay, PCR primers PF2 and PR1 (Table 1) were designed to amplify a 76-bp fragment of the *ssrA* gene. PF2 primer was located at positions 179–198 bp and PR1 was located at position 237–254 bp of the *P. aeruginosa* PAO1 *ssrA* gene (GenBank ID:NC_002516). Primers for the *Burkholderia* genus-specific diagnostics assay, BF1 and BR1, were designed to amplify a 139-bp region of the *rnpB* gene in all *Burkholderia* species BF1 primer was located at positions 1–18 bp and BR1 was located at position 122–139 bp of the *B. cenocepacia* HI2424 *rnpB* gene (GenBank ID: NC_008542). Oligonucleotide primers and probes were supplied by *Eurofins* MWG Operon (Ebensburg, Germany) or Integrated DNA Technology (Leuven, Belgium).

Conventional PCR

Conventional PCR was performed using the sequencing primers outlined in Table 1 on the iCycler iQ thermal cycler (Bio-Rad, Hercules, CA, USA). All reactions were carried out in 0.2-ml PCR tubes, containing 2.5 µl 10× buffer (15 mM $MgCl_2$), forward and reverse primers (0.2 µM final conc.), 0.5 µl dNTP mix (10 mM: deoxynucleoside triphosphate set, Roche Diagnostics, Basel, Switzerland), 0.5 U Taq DNA polymerase (Roche Diagnostics), 1 µl of template DNA and adjusted to a final volume of 25 µl with the addition of nuclease-free water (Applied Biosystems, Carlsbad, CA, USA). The thermal cycling parameters consisted of an initial denaturation step at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C (30 s), amplification at 50 °C (30 s), and extension at 72 °C (30 s), followed by a final elongation at 72 °C for 7 min.

Nucleic acid sequencing

Nucleotide sequence data used for real-time PCR diagnostics assay design was obtained either from publicly available databases or were generated in this study. Sequencing primers (Table 1) were designed to amplify ~329 bp of the *ssrA* gene of *Pseudomonas* species and 327–349 bp of the *rnpB* gene of *Burkholderia* species. Conventional PCR products were generated as described above, followed by purification using the High Pure PCR product purification kit (Roche Diagnostics). The purified PCR products were sequenced externally (Sequiserve, Vaterstetten, Germany).

Development of an internal amplification control for real-time PCR

An internal amplification control (IAC) was developed using a modified version of the composite primer approach as previously described [21, 22, 40]. The IAC was based on a real-time PCR diagnostics assay previously developed in this laboratory to specifically detect the *ALS1* gene of *Candida albicans* [41]. Composite primers CIAC F and CIAC R (containing nucleotide sequence of both *Candida albicans ALS1* gene and the *aqualysin 1* gene from *Thermus aquaticus*) were used to amplify a chimeric PCR product by conventional PCR.

The PCR product was cloned into a plasmid (pCR4 TOPO) using the TOPO TA cloning kit (Invitrogen) as per the manufacturer's guidelines. Plasmid DNA was then extracted and purified from a clone using the QIAprep Spin Miniprep Kit (Qiagen, Germany) as per the manufacturer's guidelines. To confirm the extracted plasmid contained the correct insert, prior to use in real-time PCR, a PCR product was generated and sequenced. In a real-time PCR this IAC was amplified using *T. aquaticus*-specific primers but the probe detected a segment of the *C. albicans ALS1* gene.

Real-time PCR

Real-time PCR diagnostics assays were initially tested in a monoplex format to determine the specificity and analytical sensitivity. During this initial testing, the probes for the *P. aeruginosa* and *Burkholderia* genus assays were both labeled with FAM and Black Hole Quencher 1 (BHQ1). The IAC assay probe was labeled with TYE665 and Iowa Black RQ-Sp (IBRQSp). To allow for multiplexing of the diagnostics assays, the *P. aeruginosa*-specific probe was subsequently labeled with HEX and BHQ1. The multiplex real-time PCR assay was optimized and the specificity and analytical sensitivity determined.

In order to demonstrate specificity of the designed multiplex assay, genomic DNA from 77 microorganisms (Supplementary Tables S1 and S2) was isolated as described in DNA isolation and quantification and added individually to the real-time PCR at a concentration of 10^4 genome equivalents (GE) per reaction. The analytical sensitivity of each assay in multiplex format was established using probit regression analysis. The limit of detection (LOD) was also investigated in the presence of a background of pooled DNA from ten other bacteria. The final concentration of the pooled bacterial genomic DNA was 10^4 GE per reaction. These bacteria were *Enterobacter aerogenes* NCTC 10006, *Staphylococcus aureus* DSM 21705, *Proteus mirabilis* DSM 4479, *Acinetobacter baumannii* DSM 30007, *Klebsiella pneumonia* DSM 9377, *Stenotrophomonas maltophilia* DSM 50170, *Serratia marcescens* DSM 1608, *Sphingomonas paucimobilis* LMG 2239, *Ralstonia pickettii* DSM 6297, *P. aeruginosa* DSM 19880, and *B. cepacia* DSM 7288.

Monoplex real-time PCR was performed in a 20-µl reaction volume on the LightCycler 2.0 instrument using the LightCycler TaqMan Master kit (Roche Diagnostics). Each monoplex reaction contained 5× master mix (4 µl), forward and reverse primer (0.5 µM final conc.), probe (0.2 µM final conc.), 0.5 U uracil-DNA glycosylase (UDG) (New England Biolabs, Ipswich, MA, USA), 5 % dimethyl sulphoxide (DMSO), template DNA (2 µl) and the volume adjusted to 20 µl with the addition of nuclease-free water. The thermal cycling parameters consisted of 10 min at 37 °C to incubate UDG followed by 10 min incubation at 95 °C to deactivate UDG, activate the polymerase, and denature the template DNA. Subsequently, 50 cycles of 95 °C for 10 s and 60 °C for 30 s were performed, with a final cooling step at 40 °C for 10 s. The temperature transition rate for all cycling steps was 20 °C/s.

Multiplex real-time PCR reactions were subsequently performed on the LightCycler 480 instrument using the LightCycler 480 probes master kit (Roche Diagnostics). Each multiplex reaction contained 2× master mix (20 µl), forward and reverse primer (0.5 µM final conc.) FAM, HEX, and TYE665 probes (0.2 µM final conc.), UDG (0.8 U), DMSO (5 %). Template DNA (10 µl) and IAC DNA (1,000 recombinant plasmid copies in 2 µl) were added to each reaction mix. Nuclease-free water was added to a final volume of 40 µl. A no-template control (NTC) reaction was included in each experiment. The cycling parameters used for multiplex reactions were in accordance with monoplex parameters. The temperature transition rate, referred to as the ramp rate on the LightCycler 480, was 4.4 °C/s while heating and 2.2 °C/s on cooling. To avoid fluorescence leakage between channels a color compensation file was generated prior to experimental analysis as outlined by the manufacturer [3].

Generation of standard curves

An overnight culture of either *P. aeruginosa* or *B. cepacia* grown in R2A broth with an optical density at 600 nm (OD_{600}) of 0.1 was serially diluted tenfold (seven dilutions) in sterile phosphate buffered saline (PBS) (Oxoid). One hundred µl of each dilution was spread on R2A agar and incubated for 24 h at 30 °C. These plates were used to count colony-forming units (CFU). Aliquots (100-µl) from each dilution were centrifuged at 18,000 × *g* for 10 min and the supernatant was gently removed. The cells were resuspended in 180 µl of Buffer ATL (DNeasy Blood and Tissue kit, Qiagen). Subsequently, steps 2–8 of the purification of total DNA from animal tissue procedure were followed according to the manufacturer's guidelines. DNA was eluted in 200 µl of sterile nuclease-free water. The final DNA elutions were evaporated (80 °C for ~90 min) and the DNA resuspended in 10 µl of sterile nuclease-free water. This was then used as template DNA in the multiplex real-time PCR reaction. Standard curves for both *P. aeruginosa* and *B. cepacia* were included for the simultaneous detection and quantification of both bacteria. Standard curves were derived by plotting threshold cycle (C_T) values against overnight plate counts [CFU ($\log_{10}$)].

Detection and quantification of *P. aeruginosa* and *B. cepacia* in HPW

Initially, sterile nuclease-free water (200 ml, pH 5.5–7.5, temperature 18–22 °C) (Invitrogen) was spiked with approximately 10–10^3 cfu/100 ml (overnight culture serially diluted in PBS) of *P. aeruginosa* DSMZ 19880 or *B. cepacia* DSMZ 7288 (determined through plate counts, data not shown) and mixed thoroughly. Sterile nuclease-free water was also simultaneously spiked with low concentrations (10^2–2×10^2 cfu/100 ml) of *B. cepacia* and *P. aeruginosa* and assessed as outlined below. To further demonstrate the robustness of the method developed, low concentrations of *B. cepacia* (10^2–2×10^2 cfu/100 ml) were also tested for in the presence of high-level *P. aeruginosa* (10^3–2×10^3 cfu/100 ml) and vice versa. Samples were then filtered using 0.45-µm, 25-mm cellulose nitrate/acetate membrane filters (Millipore, Bedford, MA, USA). Each filter was placed into a sterile 1.5-ml microcentrifuge tube, suspended in sterile PBS and then vortexed. Mild sonication at (37 Hz, 1 min, 22 °C) was carried out to detach cells from the filter membrane. After sonication, the resuspended cells were centrifuged at $12{,}000 \times g$ for 15 min and the supernatant was gently removed. The cells were resuspended in 180-µl of Buffer ATL (DNeasy Blood and Tissue kit, Qiagen) followed by steps 2–8 of the purification of total DNA from animal tissue procedure according to the manufacturer's guidelines. The DNA was eluted in 200 µl of sterile nuclease-free water. The final DNA elution was evaporated and the DNA resuspended in 10 µl of sterile nuclease-free water. This was then used as template DNA in the multiplex real-time PCR reaction. The level of *P. aeruginosa* and *B. cepacia* in the water samples was quantified by comparison to one of the internal standard curves, generated as described above. A negative control of 200 ml of sterile nuclease-free water spiked with 200 µl of sterile PBS was included for each set of filtration experiments described above.

Results

Diagnostics target identification

The diagnostics target used in this study for the specific detection of *P. aeruginosa* is a region of the *ssrA* gene. In silico analysis revealed that this gene exhibits highly conserved nucleotide sequences at its 5′ and 3′ ends. However, significant intragenic nucleotide sequence variation between these conserved flanking regions was observed, permitting the design of a specific *P. aeruginosa* probe. Based on in silico analysis, it was determined that the *ssrA* gene has greater nucleotide sequence heterogeneity between *P. aeruginosa* and closely related microorganisms compared to the other bacterial genes evaluated in this study, including the 16S rDNA, *lepA*, and *rnpB* genes (data not shown).

For the specific detection of the *Burkholderia* genus, another non-coding RNA gene, the *rnpB* gene, which exhibits highly conserved intragenic regions within members of the *Burkholderia* genus, was chosen as a diagnostics target for this genus. Initial in silico analysis demonstrated sufficient nucleotide sequence similarity within this gene to allow for the design of a specific probe for the collective detection of this genus. Moreover, this gene showed greater nucleotide sequence heterogeneity between members of the *Burkholderia* genus and closely related microorganisms compared to other genes evaluated including the 16S rDNA, *lepA*, and *ssrA* genes (data not shown).

Internal amplification control

The IAC developed in this study used a non-competitive approach. Therefore, in order for a result to be considered valid using this multiplex real-time PCR diagnostics assay, a positive signal must be obtained for all samples except the no-template control (NTC) in the TYE665 detection channel on the LightCycler 480. This eliminates the reporting of false-negative results, which may be due to PCR inhibition in sample, thermocycler malfunction, or problems with assay reagents. If any of the assay targets are not detected in the IAC channel, the result is considered invalid and must be repeated [22]. The IAC designed in this study is a unique chimeric PCR product incorporated into a plasmid. It is spiked into the PCR mix to act as an internal control target. The IAC target combines both *T. aquaticus* and *C. albicans* nucleotide sequence. As this nucleotide sequence does not occur in nature, the construct eliminates the risk of this component of the real-time PCR assay cross reacting with nucleotide sequences of naturally occurring microorganisms. The IAC, at a concentration of 1,000 plasmid equivalents per reaction, was detected in all samples tested (Supplementary Fig. S1 C).

Specificity of the diagnostic assays

The specificity of each real-time PCR diagnostics assay was confirmed both in monoplex and multiplex formats using the specificity panel listed in Supplementary Tables S1 and S2. The *P. aeruginosa*-specific real-time PCR diagnostics assay detected the 14 *P. aeruginosa* strains in our collection (as shown by the representative real-time amplification curves in Supplementary Fig S1A). In silico analysis of the nucleotide sequence of this probe region

shows it is conserved in the 16 other strains of *P. aeruginosa* for which nucleotide sequence data is available (Supplementary Table S3). The remaining 12 *Pseudomonas* species and 51 other microorganisms tested in this study were not detected using the *P. aeruginosa* specific real-time PCR diagnostics assay.

Ten *Burkholderia* species in our collection were tested using the *Burkholderia* genus specific real-time PCR diagnostics assay (Supplementary Figure S1B). All ten *Burkholderia* species were detected and none of the remaining 67 microorganisms (Supplementary Table S1 and S2) tested were detected. Based on in silico analysis, this specific probe region is conserved in the 71 other *Burkholderia* species/strains for which nucleotide sequence data are available (Supplementary Table S3). The specificity of the IAC assay was also tested using the same panel of microorganisms and no cross reactivity was observed.

Analytical sensitivity of the diagnostics assays

The limit of detection (LOD) of each diagnostics assay was evaluated in both monoplex and multiplex real-time PCR formats. Total genomic or plasmid DNA was quantified and serial dilutions were prepared from 10^6 to 0.1 genome/plasmid equivalents. Supplementary Fig. S1 and S2 show the analytical sensitivity of the *P. aeruginosa*-specific real-time PCR diagnostics assay in monoplex format. The genome size of *P. aeruginosa* PAO1 (DSMZ 19880) is 6.26 MB (GenBank ID: NC_002516), which equates to approximately 6.8 fg of DNA per cell [56]. *B. cepacia* (DSMZ 7288) was used for sensitivity testing of the *Burkholderia* genus assay. It has a genome size of 8.1 MB, which equates to approximately 8.8 fg of DNA per cell [46]. The IAC plasmid including PCR insert is 4,174 bp (approximately 4.55 ag of DNA per plasmid copy). The dilution series was run in triplicate for each real-time PCR diagnostics assay. In monoplex format, all three diagnostics assays had an analytical sensitivity of 1 GE. In multiplex format, the LOD was established using probit regression analysis. With 95 % confidence, the analytical sensitivities of the *P. aeruginosa* and the *B. cepacia* assays are 3.5 GE and 3.7 GE, respectively. The LOD for each assay was not affected when investigated in the presence of a background of pooled DNA from ten other microorganisms at a concentration of 10^4 GE per reaction. The IAC, at a concentration of 1,000 genome equivalents per reaction, was included in all samples during sensitivity testing and was detected at C_T values from 27.2 to 28.6. The amplification efficiency (E) of the multiplex real-time PCR reactions ranged from 1.92 to 2.02.

Diagnostics method for the detection and quantification of bacterial contamination of HPW

HPW samples (200 ml) were spiked with *P. aeruginosa* and/or *B. cepacia* at a concentration ranging from 10 to 2×10^3 cfu per 100 ml and the sample was filtered. Total genomic DNA was extracted from each filter and tested using the multiplex real-time PCR diagnostics assay developed in this study. Bacterial contamination was quantified by comparison to the internal standard curves. The dynamic range for both standard curves used in this study was 10^2–10^7 cfu/reaction. From this analysis, it was determined that it is possible to reliably detect and identify the spiked bacteria at a concentration of 1.06×10^2 cfu/100 ml for *P. aeruginosa* and 2.66×10^2 cfu/100 ml for *B. cepacia* when spiked individually. When both bacteria were spiked simultaneously, it was possible to detect a spiked bacterial load of 1.80×10^2 cfu/100 ml for *P. aeruginosa* and 2.70×10^2 cfu/100 ml for *B. cepacia* from a 200-ml filtered HPW sample. For complete results of this analysis, see Table 2.

Table 2 Results of the detection and quantification of contamination with *P. aeruginosa* and *B. cepacia* at varying cfu per 100 ml of HPW

	P. aeruginosa cfu/100 ml	*B. cepacia* cfu/100 ml	Plate count cfu/100 ml (SD)	C_T value (SD)	Mean calculated cfu/100 ml from real-time PCR (SD)
P. aeruginosa	10^2	0	92 (12)	36.14 (0.38)	106 (57)
P. aeruginosa	10^2	10^3	126 (9)	37.05 (0.23)	134 (20)
P. aeruginosa	10^3	10^2	1,260 (90)	33.14 (0.11)	1,470 (93)
P. aeruginosa	10^2	10^2	156 (7)	36.69 (0.76)	180 (76)
B. cepacia	0	10^2	176 (22)	34. 98 (0.42)	266 (74)
B. cepacia	10^3	10^2	208 (16)	35.43 (0.36)	336 (153)
B. cepacia	10^2	10^3	1,870 (160)	33.73 (0.22)	2,174 (406)
B. cepacia	10^2	10^2	187 (16)	35.87 (0.42)	270 (161)

Bacteria spiked at a concentration of 10^x–2×10^x cfu/100 ml

SD standard deviation

Discussion

The novel multiplex real-time PCR diagnostics assay described in this study uses NAD targets for the specific quantitative detection and identification of *P. aeruginosa* and the *Burkholderia* genus. Studies have indicated that *P. aeruginosa* and members of the *Burkholderia* genus are among the most common pathogenic contaminants of HPW [26, 38, 43]. These microorganisms are also significant hospital-acquired infectious agents. They can persist in hospital environmental reservoirs and also have the ability to colonize medical devices and patient-care equipment [23, 47, 57].

The molecular target used to specifically detect *P. aeruginosa* was a region of the *ssrA* gene. The *ssrA* gene encodes the high copy number tmRNA and is present in all sequenced bacterial genomes to date [37]. The gene exhibits intragenic nucleotide sequence heterogeneity between bacterial species, making this gene a valuable target for use in NAD assays [48]. Further examples of the usefulness of this nucleic acid diagnostics target has been successfully demonstrated with the development of specific NAD assays to detect a bacterium of interest, utilizing both direct and in vitro amplification detection technologies [11, 35, 40, 54].

The second diagnostics target selected for the detection and identification of the *Burkholderia* genus was a region of the *rnpB* gene. This gene encodes the high copy number M1 RNA and is present in all Prokaryotes, Archaea, and Eukaryotes sequenced to date [16, 19]. Multiplex real-time PCR diagnostics assays have also been described using the *rnpB* gene, highlighting its suitability as a diagnostic target [24, 29].

NAD technologies such as real-time PCR are frequently used in the environmental sector to detect contaminating microorganisms in water. A significant advantage of using multiplex real-time PCR to detect microbial contamination in water is that it can be completed within one working day. Another advantage is that it permits not only the rapid and sensitive detection of contaminating microorganisms but also the identification of the microorganism without the requirement for further testing.

To our knowledge, this is the first report of an internally controlled triplex assay for the quantitative detection and identification of *P. aeruginosa* and *B. cepacia* from HPW. A previous study by Lee et al. [31] demonstrated detection of *P. aeruginosa* from water with sensitivities of 3.3×10^2–2.3×10^3 CFU per PCR in a monoplex format. Despite the complexity of the multiplex internally controlled assay described in this current study, the LOD is comparable to that of Lee et al. and other studies that use DNA-based in vitro amplification technologies for the rapid detection of microorganisms from water [31, 39, 51]. Using the developed multiplex real-time PCR diagnostics assay, it was experimentally determined that it was possible to directly detect a spiked bacterial load of 1.06×10^2 cfu/100 ml for *P. aeruginosa* and 2.66×10^2 cfu/100 ml for *B. cepacia* from a 200-ml filtered HPW sample when spiked individually. Furthermore, when both bacteria were spiked simultaneously it was possible to detect a spiked bacterial load of 1.80×10^2 cfu/100 ml for *P. aeruginosa* and 2.70×10^2 cfu/100 ml for *B. cepacia* from a 200-ml filtered HPW sample. These results further demonstrate the robustness of the diagnostics methodology developed.

In addition, the detection sensitivities attained in this study were achieved without culture pre-enrichment of the HPW sample. The ability to detect contaminating microorganisms without culture pre-enrichment is a major advantage as it greatly reduces the time to result. Traditional microbial analysis of HPW can take 5–7 days. This rapid method can quantitatively detect and identify low-level contamination with these microorganisms in approximately 4 h. This is important in the pharmaceutical sector as testing of HPW needs to be both qualitative and quantitative.

While this current study has focused on developing a rapid method for the quantitative detection and identification of *P. aeruginosa* and the *Burkholderia* genus, a number of other microorganisms have also been reported as contaminants of HPW, medical devices, and patient-care equipment [5, 7, 30]. As such, work has begun in this laboratory on developing a multiparametric approach for the quantitative detection and identification of a these common bacterial contaminants based on the methodology presented here.

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Conflict of interest The authors declare that they have no conflicts of interest.

Ethical standards All experiments performed during this study comply with the current Irish laws.

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