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ORIGINAL PAPER

Evaluation of molecular techniques for identification and enumeration of *Raoultella terrigena* ATCC 33257 in water purifier efficacy testing

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Abstract *Raoultella terrigena* ATCC 33257, a representative of the coliform group, is commonly used as a challenge organism in water purifier efficacy testing. In addition to being time consuming, traditional culturing techniques and metabolic identification systems (including automated systems) also fail to accurately differentiate this organism from its closely related neighbors belonging to the Enterobacteriaceae group. Molecular-based techniques, such as real-time quantitative polymerase chain reaction (qPCR) and enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting, are preferred methods of detection because of their accuracy, reproducibility, specificity, and sensitivity, along with shorter turnaround time. ERIC-PCR performed with the 1R primer set demonstrated stable unique banding patterns (~800, ~300 bp) for *R. terrigena* ATCC 33257 different from patterns observed for *R. planticola* and *R. ornithinolytica*. The primer pair developed from *gyraseA* (*gyrA*) sequence of *R. terrigena* for the SYBR Green qPCR assay using the AlleleID® 7.0 primer probe design software was highly specific and sensitive for the target organism. The sensitivity of the assay was 10^1 colony forming units (CFU)/ml for whole cells and 4.7 fg with genomic DNA. The primer pair was successful in determining the concentration ($5.5 \pm 0.3 \times 10^6$ CFU/ml) of *R. terrigena* from water samples spiked with equal concentration of *Escherichia coli* and *R. terrigena*. Based on these results from the ERIC-PCR and the SYBR Green qPCR assay, these molecular techniques can be efficiently used for rapid identification and quantification of *R. terrigena* during water purifier testing.

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Introduction

Raoultella terrigena ATCC 33257 is most commonly selected as a challenge organism representative of the coliform group to test the efficacy of water purification systems. The guide standard was developed to validate the microbial disinfection claims of treatment systems using chlorine, iodine, filtration or ultraviolet (UV) irradiation. In 1987, the US Environmental Protection Agency (USEPA) taskforce developed compliance requirements and methods for verification (the Guide Standard and Protocol for Testing Microbiological Purifiers) [25]. According to the standard method, challenge organism (*R. terrigena*) recovery is accomplished using different media such as mEndo and nutrient agar. However, none of these media are selective for *R. terrigena*. mEndo is partially selective, but all coliforms appear as red colonies with metallic sheen. Several challenges are encountered by the testing laboratory when using nonselective media for recovery and identification of *R. terrigena*. Test units, by nature, are nonsterile environments, so there could be several background organisms competing on the recovery plates. Coliforms should not be present in the test unit, but their presence cannot be ruled out, as some manufacturers perform pretesting in their own facility. Reagents used in challenge water preparation may also contain background organisms that can interfere with analysis. Occasionally, noncoliforms produce colonies with typical sheen, and coliform organisms may also produce atypical colonies [6, 18].

Raoultella terrigena was first described in 1981 [13] as *Klebsiella terrigena* and was reclassified under the new

genus of *Raoultella* in 2001 [8] along with *Raoultella* (*Klebsiella*) *planticola* and *Raoultella* (*Klebsiella*) *ornithinolytica* based on phylogenetic relationship. In microbiology laboratories, *Klebsiella* and *Raoultella* strains are identified using conventional methods based on biochemical tests [2]. Automated instruments such as Biolog, VITEK, and API systems also fail to identify some of these bacteria to species level because of their similar biochemical profile owing to their high similarity and homology at genomic level [11, 15, 27]; for example, *R. ornithinolytica* is usually misidentified as *R. terrigena* or *R. planticola* and *R. terrigena* as *K. pneumoniae* or *K. oxytoca* when using automated identification systems [14, 17].

Molecular techniques based on polymerase chain reaction (PCR) have been successfully developed and implemented for detection and enumeration of different microorganisms (pathogenic and nonpathogenic) from a wide variety of environments [7, 9, 21, 22]. Molecular-based methodologies have high reproducibility, accuracy, and specificity for recognition of minimal differences at genomic level for highly related organisms such as those belonging to the *Klebsiella* (*Raoultella*) group [10]. These techniques not only reduce the turnaround time but also overcome the limitations associated with conventional tests. Previous studies have utilized housekeeping gene (16S rRNA, *rpoB*, *gyrB*, *gyrA*) sequences to infer the phylogenetic relationship of the *Klebsiella* group [4, 5, 8]. However, sequencing is not always a feasible technique for routine analysis of these organisms in a microbiology testing laboratory, as it requires special instrumentation and technical skills. To overcome these problems there is a need for implementation of more robust, accurate, and time-efficient methods such as DNA fingerprinting and real-time quantitative PCR (qPCR) for detection of these bacteria. Both DNA fingerprinting and qPCR have been successfully used in diagnosis of infectious diseases in clinical laboratory. Granier et al. [10] performed ERIC-1R PCR assay for identification of *Raoultella* sp. identified as *K. oxytoca* in clinical laboratory. Hartman et al. [12] and Sun et al. [23] developed qPCR assays for rapid detection of *K. pneumoniae* in clinical specimen and infant formula.

In view of the above-mentioned challenges, the objectives of this study are to (1) evaluate ERIC-1R PCR fingerprinting and (2) develop qPCR assay based on SYBR Green chemistry targeting a region within the housekeeping gene *gyraseA* (*gyrA*) for rapid and accurate identification and enumeration of *R. terrigena* ATCC 33257 used as challenge organism in water purifier testing. The bacterial enzyme DNA gyrase (topoisomerase II) catalyzes the negative supercoil into covalent closed circular DNA and consists of two subunits. Subunit A is encoded by the *gyrA* gene [19]. Previous studies have reported *gyrA* as a suitable molecular chronometer for differentiation of closely related organisms that cannot be separated using 16S rRNA sequencing [5].

Materials and methods

Bacterial strains

The following bacterial type strains included in this study were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA): *Raoultella terrigena* ATCC 33257^T, *R. planticola* ATCC 33531^T, *R. ornithinolytica* ATCC 31898^T, *Escherichia coli* ATCC 11229 and 25922, *Klebsiella pneumoniae* ATCC 13883 and 4352, *Pseudomonas fluorescens* ATCC 13525, and *P. aeruginosa* ATCC 9027. For ERIC-PCR, all strains were grown on tryptic soy agar (TSA) and tryptic soy broth (TSB) (BD, Franklin Lakes, NJ, USA) for 24 h at 37°C. For qPCR, the bacterial strains except for *P. fluorescens* were grown in TSB and incubated at 37°C for 24 h to achieve the necessary cell density. The colony forming units (CFU)/ml of *R. terrigena* for qPCR was estimated by culturing samples on TSA by spread plating technique [3] and incubating the plates at 37°C for 24 h. Serial dilution for spread plating was performed in both phosphate buffer and 0.85% NaCl solution. The CFU/ml was used to plot the qPCR standard graph by determining the relation between CFU and amount of DNA extracted, assuming that all cells were viable as they were taken from the log phase of growth.

Whole-cell preparation for ERIC-PCR and qPCR

For ERIC-PCR, one or two colonies of approximately 0.3 mm diameter were completely picked from TSA plate (24 h culture) and were suspended in 300 µl nuclease-free water in a 1.5-ml microcentrifuge tube. The microcentrifuge tube was vortexed for 30 s. The microcentrifuge tube was centrifuged at 15,000 × *g* for 5 min to obtain cell pellet. The supernatant was discarded, and the pellet was resuspended in 500 µl nuclease-free water. The microfuge tube was heated in a dry bath at 100°C for 10 min. After 10 min of heating, the tube was immediately chilled in crushed ice for at least 5-10 min until further use. Before quantification of the extracted DNA, the microfuge tube was centrifuged at 10,000 × *g* for 2 min to remove cell debris and other contaminants. The supernatant containing the DNA was placed in a sterile microfuge tube and quantified using NanoDrop® 1000 spectrometer (Thermo Scientific, Wilmington, DE, USA). The ratio of absorbance at 260 and 280 nm was used to assess the purity of the extracted DNA. A ratio in the range of approximately 1.7 and 2.0 was used for the PCR assays. The DNA sample was diluted to stock solution of 20 ng/µl.

For qPCR, the same procedure was used for extraction of DNA from well-isolated colonies. The well-isolated colonies were used to determine the specificity of the assay in cultures grown in solid media. The DNA sample was diluted to stock solution of 10 ng/μl. However, for the sensitivity assay (24-h-old culture) and construction of standard graph (log-phase culture), DNA was extracted from whole cells of liquid cultures. From 24-h-old culture and log-phase culture growing in TSB, 1 ml was taken in a sterile microfuge tube, and the above-mentioned procedure was followed to extract the DNA. After chilling the tube in ice for 5 min, the tube was centrifuged at 10,000 × *g* for 2 min, and 10-fold serial dilutions (five series) were performed by diluting 10 μl extracted DNA in 90 μl nuclease-free water. DNA was quantified using NanoDrop. Sensitivity assay was performed to determine the limit of detection, and the standard graph was used for enumeration of *R. terrigena* ATCC 33257 from samples.

ERIC-PCR

ERIC-PCR was performed using the primer ERIC-1R (5′ ATG TAA GCT CCT GGG GAT TCA C 3′) described previously [26]. ERIC-PCR was performed under two different conditions. The two different reaction conditions were used to compare the banding patterns, resolution, and reproducibility of the bands obtained under different conditions.

One of the reactions was carried out using Ready-To-Go RAPD™ analysis beads (GE Healthcare, Buckinghamshire, UK). Each bead contains buffer, dATP, dCTP, dGTP, dTTP, bovine serum albumin, AmpliTaq, and Stoffel fragment. The reaction was performed according to the manufacturer's instructions using 2.5 μl ERIC-1R primer (from 10 μM stock). The *E. coli* BL21 (DE3) DNA provided with the kit was used as positive control.

The second PCR reaction was performed by taking 25 μl Hotstar Taq Polymerase Master Mix (Qiagen, Valencia, CA, USA), 5 μl $MgCl_2$ (from 25 mM stock), 2.5 μl ERIC-1R primer (from 10 μM stock), 1.5 μl DNA template (from 20 ng/μl stock), and 16 μl nuclease-free water for a total of 50 μl PCR reaction volume per sample per tube.

PCR program for ERIC-PCR

PCR reactions were carried out using the Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA). For the first reaction condition, the following temperature control option was used: initial denaturation at 95°C for 5 min; followed by 45 cycles of denaturation at 95°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min; and a final extension step at 72°C for 7 min.

The second PCR reaction was performed using the temperature control of initial denaturation at 95°C for 15 min; followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 2 min; and a final extension step at 72°C for 10 min.

Gel electrophoresis

Following PCR, the reaction mixture containing the PCR product was electrophoresed in a 1.2% and 2% premade agarose E-Gel cassette (Invitrogen, Carlsbad, CA, USA) stained with ethidium bromide. Electrophoresis was carried out for 30 min according to the manufacturer's instructions. Gels were photographed under ultraviolet light using Kodak gel documentation system (Kodak, Rochester, NY, USA). To confirm the reproducibility of the technique, each reaction was performed in duplicate per isolate. The DNA fingerprint obtained for each isolate was subjected to visual inspection and interpretation.

Analysis of *gyrA* sequence and design of real-time SYBR Green I primer pair

The *gyrA* gene sequences of all the closely related species belonging to the genera *Klebsiella* and *Raoultella* were obtained from National Center for Biotechnology Information (NCBI) database. The *gyrA* sequence of *R. terrigena* was analyzed along with the other closely related species using Basic Local Alignment Search Tool (BLAST) and CLUSTAL X [24] to determine sequence similarities. The primer probe design software Allele ID 5.0 (Premier Biosoft, Palo Alto, CA, USA) was used to design the SYBR Green I primer pair specific for *R. terrigena* targeting a particular region of the *gyrA* gene. The sequences of the primer pair are as follows: forward primer (RT1_F) 5′ T TCGTTGATAACTATGACGGCACG 3′ (24 bp) and reverse primer (RT2_R) 5′ GTTTACCAGCAGGTT GGGGATT 3′ (22 bp). The theoretical specificity of the primer pair was evaluated using Primer3 and BLAST [1, 20]. The size of the amplicons was 75 bp. The primer pair was synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA).

qPCR assays were performed using SensiMix™ SYBR & Fluorescein Kit (Bioline, Taunton, MA, USA). Each 50 μl reaction consisted of the following: 25 μl SensiMix, 10 μl genomic DNA (10 ng/μl stock), 2 μl each of forward and reverse primer (5 μM working stock), and 11 μl nuclease-free water. The PCR program used for the qPCR assay was as follows: cycle 1: (1×), step 1, 95°C for 10 min; cycle 2: (35×), step 1, 95°C for 15 s, step 2, 65°C for 10 s; cycle 3: (1×), step 1, 95°C for 1 min; cycle 4: (1×), step 1, 55°C for 1 min; cycle 5: (80×), step 1, 55°C for 10 s; increase setpoint temperature after cycle 2 by 0.5°C. For sensitivity assays, different concentrations of DNA were used and PCR cycle was enabled for 45× instead of 35×.

Each reaction was performed at least in duplicate. Melting curve analysis was performed to detect the presence of any nonspecific product or primer-dimer formation. Agarose gel electrophoresis was also performed to confirm the presence of the specific PCR product. The sensitivity of the assay was also tested in presence of a high concentration (10^6 CFU/ml) of a nonspecific target such as *R. planticola* and *R. ornithinolytica* mixed with low concentration (10^3 CFU/ml) of *R. terrigena*.

Spike sample test

Water samples were spiked with (1) known concentrations [10^4–10^6 colony forming units (CFU)/ml] of *R. terrigena* only and (2) equal concentrations (1:1) of *E. coli* and *R. terrigena* culture. In both instances, spiked samples were diluted with 0.85% NaCl solution to perform spread plating on TSA. The samples were also used for enumeration by qPCR analysis.

Results and discussion

ERIC-PCR

ERIC-PCR fingerprinting was performed for differentiation and identification of *R. terrigena* ATCC 33257. The ERIC-1R PCR profile demonstrated different fingerprint patterns for each of the bacterial strains tested. *Raoultella terrigena* ATCC 33257 demonstrated a unique fingerprint profile clearly different from its closely related neighbors *R. planticola* ATCC 33531 and *R. ornithinolytica* ATCC 31898, which are difficult to distinguish based on biochemical tests [14]. There were two signature bands (~800 and ~300 bp) for *R. terrigena* observed under both reaction conditions (Fig. 1a). Two different PCR conditions were used to generate comparative profiles so that the condition generating better resolution and banding pattern could be used for identification purpose. The results were consistent with previous findings [10]. However, the moderate-intensity band of ~2,100 bp observed by Grainer et al. [10] was absent in the present study, whereas a second high-intensity band at ~800 bp was observed using the first PCR condition. It was also observed that the first PCR condition provided better resolution and banding pattern compared with the second PCR condition (Fig. 1b). Therefore, it was evident from the present study that annealing temperature, Taq polymerase, and $MgCl_2$ concentration play a critical role in ERIC-PCR fingerprinting. However, the purity of genomic DNA did not play any part in the resolution of the fingerprint profile. ERIC-PCR was performed by three different laboratory technicians to investigate if the technique was reproducible between personnel. The technique did not exhibit any difference in the banding patterns when performed by different individuals in the laboratory (data not shown). Therefore, ERIC-1R PCR using Ready-To-Go RAPD™ analysis beads could be successfully used on routine basis for fast and accurate identification of *R. terrigena* in a microbiology testing laboratory. Moreover, the technique can also be utilized for routine quality control of different bacterial strains.

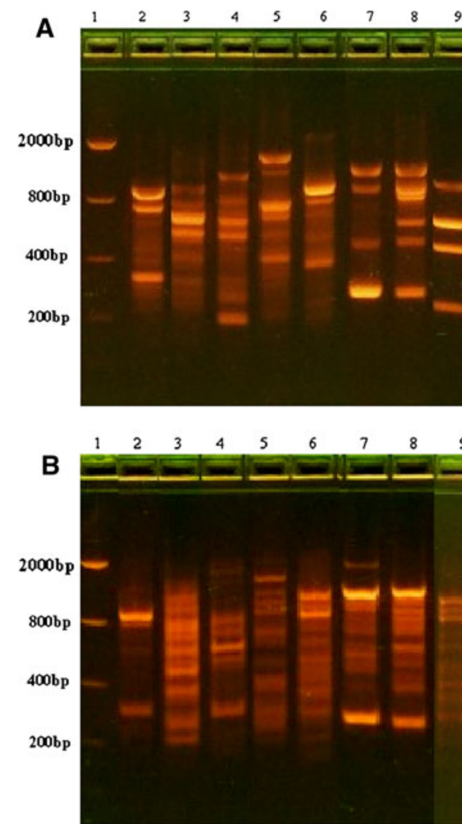

Fig. 1 E-Gel® (2% agarose) showing the ERIC-1R PCR fingerprint pattern of different Gram-negative bacteria belonging to the Enterobacteriaceae group using two different reaction conditions: **a** Ready-To-Go™ analysis beads and **b** Qiagen Hotstar Taq Polymerase Master Mix, 4 mM $MgCl_2$. *Lane 1* molecular marker (2,000 bp, 800 bp, 400 bp, 200 bp); *Lane 2 Raoultella terrigena* ATCC 33257; *Lane 3 R. ornithinolytica* ATCC 31898; *Lane 4 R. planticola* ATCC 33531; *Lane 5 Klebsiella pneumoniae* ATCC 13883; *Lane 6 K. pneumoniae* ATCC 4352; *Lane 7 Escherichia coli* ATCC 11229; *Lane 8 E. coli* ATCC 25922; *Lane 9 E. coli* BL21

Specificity of the SYBR Green qPCR assay

The specificity of the assay was determined using genomic DNA extracted from whole cells of a wide range of Gram-negative bacteria belonging to both the Enterobacteriaceae and non-Enterobacteriaceae group. Cultures from both liquid and solid media were used to test the specificity of the primer pair. Amplification of the fluorescent signals from liquid cultures was observed only for the *R. terrigena*

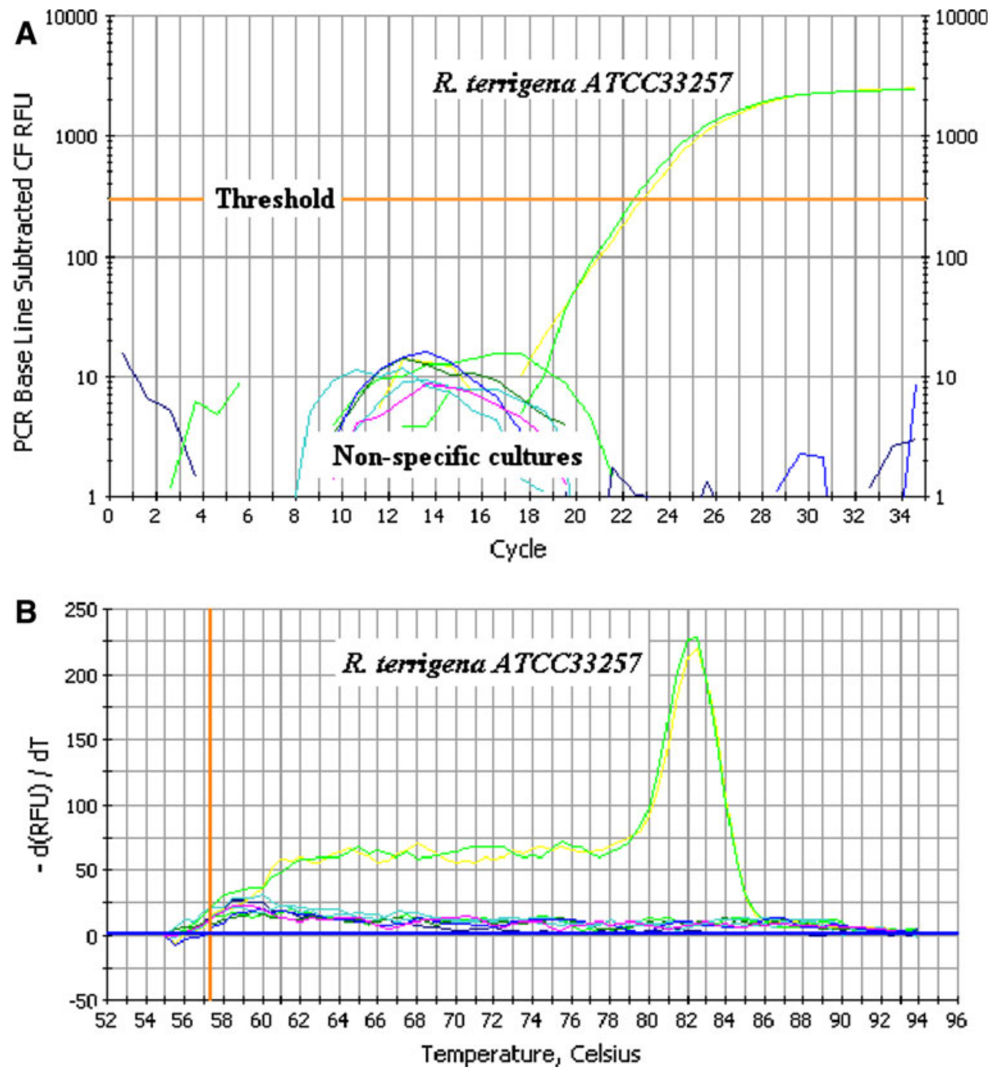

Fig. 2 Specificity assay of the SYBR Green primer pair for *Raoultella terrigena* ATCC 33257 using genomic DNA extracted from whole cells taken from liquid cultures targeting a specific region of the *gyrA* gene. **a** Logarithmic representation of the amplification signal observed only from *R. terrigena*. No amplification signal was observed from *R. planticola* ATCC 33531, *R. ornithinolytica* ATCC 31898, *Klebsiella pneumoniae* ATCC 13883 and ATCC 4352, *Escherichia coli* ATCC 11229 and ATCC 25922, *Pseudomonas fluorescens* ATCC 13525, and *P. aeruginosa* ATCC 90257. **b** Melting curve analysis indicating the presence of a single PCR product for *R. terrigena* ATCC 33257 *gyrA* gene. Each isolate was tested in duplicate

ATCC 33257 isolate with annealing temperature of 65°C for 10 s, indicating that the primer pair was highly specific for the target organism under the specific assay conditions (Fig. 2a). Similar results were observed for cultures taken from solid media plates. The assay was also capable of detecting a single colony of *R. terrigena* ATCC 33257. The threshold cycle (Ct) values for single and two colonies were 22.3 ± 0.21 and 25.3 ± 0.07, respectively (Fig. 3), indicating 10-fold difference between single and double colonies without PCR inhibition. This experiment was important as this assay will also be used for identification of *R. terrigena* from colonies growing on recovery plates against other background organisms. The melting curve analysis indicated the absence of nonspecific amplification products and primer-dimer (Fig. 2b). The melting temperature ($T_m = 82.3 \pm 0.35$°C) was consistently specific for the amplicons.

Previously developed qPCR assays using both SYBR Green and TaqMan chemistry involved DNA extraction with a commercial kit [16]. The assay developed in the present study uses whole cells, the advantage of which is the elimination of the DNA extraction step. DNA extraction using a commercial kit usually requires 1–2 h depending on the kit, whereas the whole-cell preparation requires only 15 min, thus making the assay time efficient and economical without compromising quality [21].

Sensitivity of the qPCR assay and development of the standard graph

Sensitivity was determined using genomic DNA extracted from different concentrations (10^1–10^6 CFU/ml) of whole cells of 24 h growing culture of *R. terrigena*. Amplification signals obtained for all concentrations indicated the sensitivity of the assay. The assay was able to detect as little as 4.7 femtograms (fg) of DNA, corresponding to 10 CFU/ml (data not shown). A qPCR assay using a mixture (2:1) with high concentration (1.0 ng) of nontarget DNA

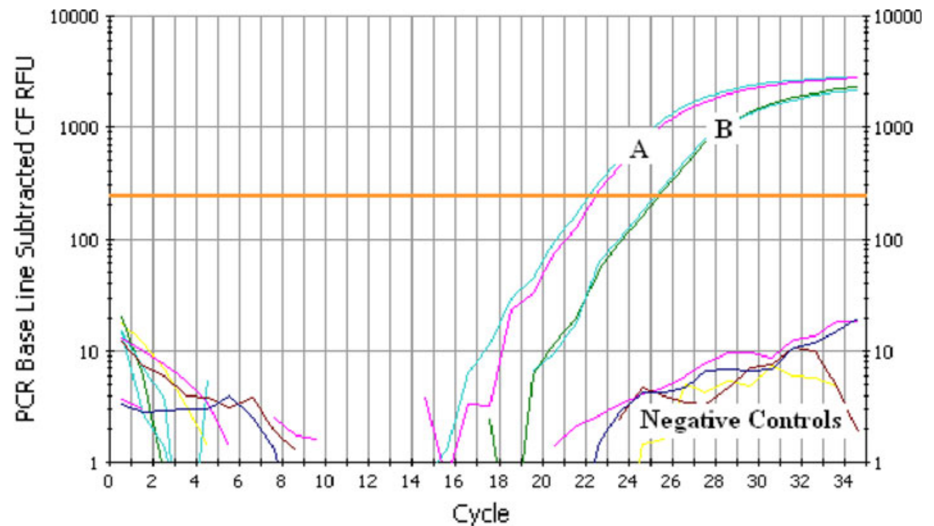

Fig. 3 Specificity assay of the SYBR Green primer pair for detection of *Raoultella terrigena* ATCC 33257 on solid medium using genomic DNA extracted from colonies. **a** Amplification signal from two colonies of a 24-h-old plate of *R. terrigena* on tryptic soy agar (TSA). **b** Amplification signal from a single colony of a 24-h-old plate of *R. terrigena* on TSA. Each sample was evaluated in duplicate

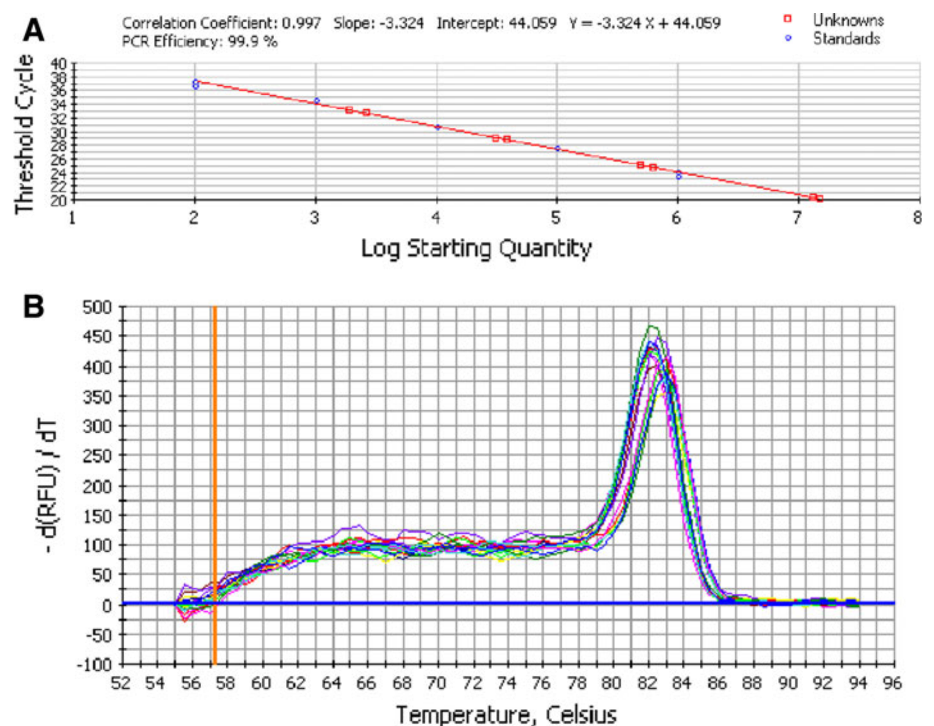

Fig. 4 Standard graph of *Raoultella terrigena* ATCC 33257 constructed using whole cells taken from liquid cultures. **a** Linear representation of the standard graph constructed using different concentrations of whole cells (10^6–10^2 CFU/ml). The primer pair targeted a region in the *gyrA* gene. Each dilution was tested in duplicate. **b** The dissociation curve, indicating the absence of any nonspecific product and primer-dimer formation

(*R. planticola* and *R. ornithinolytica*) and low concentration (0.5 ng) of target DNA (*R. terrigena*) did not exhibit any interference in the sensitivity of the assay for the target organism. All qPCR studies were supported by melting curve analysis (data not shown).

A standard curve was constructed using different concentrations of whole cells (10^2–10^6 CFU/ml) corresponding to genomic DNA (47 fg to 0.47 ng) of *R. terrigena* for the primer pair. The range of the standard curve was determined from the sensitivity (limit of detection) of the assay. Plots of cycle threshold (Ct) versus nanograms of DNA and CFU/ml of *R. terrigena* were obtained from the qPCR assay, which could be used to determine the concentration of cells present in challenge water sample. The standard graph obtained using whole cells of *R. terrigena* indicated both the linearity and the efficiency of the real-time assay, as evident from the correlation coefficient value ($R^2 = 0.997$ and slope = −3.3) (Fig. 4a). The assay was supported by melting curve analysis (Fig. 4b). The enumeration accuracy of the assay was tested using samples with known concentration of *R. terrigena*. The limit of accurate quantification of the SYBR Green qPCR assay was estimated to be 10^3 CFU/ml. A 10-fold decrease in the CFU count (from actual CFU) was observed for low concentration of cells diluted with phosphate buffer. In case of cells diluted with 0.85% NaCl, no decrease in estimation of CFU was

Table 1 Comparison of quantitative real-time PCR (qPCR) analysis and traditional plating methods for water samples spiked with *Raoultella terrigena* ATCC 33257

Sample no.	Organism	Initial concentration (CFU/ml)	*R. terrigena* count[a]		Ct value[c]
			TSA[b]	qPCR	
1	*R. terrigena*	10^6	$(5.6 \pm 0.9) \times 10^6$	$(5.7 \pm 0.3) \times 10^6$	25.0 ± 0.25
2	*R. terrigena*	10^5	$(3.5 \pm 0.5) \times 10^5$	$(3.6 \pm 0.3) \times 10^5$	29.0 ± 0.23
3	*R. terrigena*	10^4	$(2.5 \pm 0.5) \times 10^4$	$(2.1 \pm 0.2) \times 10^4$	32.9 ± 0.3
4	*E. coli*[d] + *R. terrigena*	10^6	$(3.6 \pm 0.4) \times 10^4$	$(5.5 \pm 0.3) \times 10^6$	25.8 ± 0.3

[a] All *R. terrigena* counts are in colony forming units (CFU) per ml

[b] Tryptic soy agar (TSA) was used for determining the CFU/ml

[c] Mean value of cycle threshold (Ct)

[d] 5.0×10^6 CFU/ml of *E. coli* was mixed with *R. terrigena*

observed (data not shown). The component in the phosphate buffer causing the inhibition was not determined in the present study. Further dilutions were carried out in 0.85% NaCl for all experimental purposes.

Enumeration of spike sample test

Enumeration of water samples spiked with the challenge organisms was performed to test the efficacy and the accuracy of the qPCR assay. The results did not indicate any difference in the concentration of *R. terrigena* between TSA plate counts and qPCR counts for samples spiked only with *R. terrigena*. However, for samples containing equal concentration of *E. coli* and *R. terrigena*, lower CFU/ml was observed on TSA plates for *R. terrigena* compared with qPCR analysis (Table 1). The lower *R. terrigena* colony count on TSA plates could be due to the faster generation time of the *E. coli*, which grew faster and occupied most of the surface area of the plate, inhibiting growth of *R. terrigena*.

Conclusions

This investigation confirms that molecular methods such as ERIC-PCR and qPCR provide a better identification platform for closely related species (*Klebsiella*/*Raoultella* group), which are difficult to distinguish based on differential selective media and biochemical tests. The aim of the purifier efficacy testing method is to detect the entire population of the challenge organisms present in the test system in order to ascertain the true efficiency of the purifier. Therefore, incorporation and implementation of these robust and sensitive molecular methodologies in different water testing methods will result in more accurate assessment of the microbial removal efficiency of purifiers. The significance of the SYBR Green primer pair is that it can be successfully used for species-specific identification of *R. terrigena* in water samples.

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