Propidium monoazide–quantitative polymerase chain reaction (PMA-qPCR) assay for rapid detection of viable and viable but non-culturable (VBNC) *Pseudomonas aeruginosa* in swimming pools

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Abstract

Lack of culturability in the viable but non-culturable (VBNC) bacteria and the ability to regain infectivity in favourable conditions is one of the new challenges of public health providers for *Pseudomonas aeruginosa* monitoring in environmental samples. Propidium monoazide quantitative polymerase chain reaction (PMA-qPCR) is one of the promising methods for timely detection of VBNC pathogens in environmental samples. We developed and used a method for the first time to detection of VBNC *P. aeruginosa* in swimming pool water samples using a membrane filter (MF). Moreover, the dominant model of the distribution of colonies on the MF and the effect of the culture medium and MF type on colony recovery by MF were evaluated. Swimming pool samples were subjected to conventional culture-based, qPCR and PMA-qPCR methods and the results were compared for the presence of VBNC *P. aeruginosa* in the samples. The positivity rate was 21% and 75% for *P. aeruginosa* in water samples as confirmed by standard culture-based and qPCR methods, respectively. Furthermore, of 24 samples, 9 (37.5%) were positive for VBNC *P. aeruginosa*. The developed qPCR/PMA-qPCR assay can detect the VBNC bacteria directly from aquatic samples and may result in better monitoring of recreational waters.

Keywords Colony distribution model · *gyrB* · Propidium Monoazide–quantitative polymerase chain reaction (PMA-qPCR) · *Pseudomonas aeruginosa* · Swimming pool · Viable but non-culturable (VBNC)

Introduction

Pseudomonas aeruginosa is a ubiquitous environmental opportunistic pathogen that is frequently isolated from swimming pools, hot tubs, and other recreational water environments. As a resistant bacterium to cleaning and disinfection [1], *P. aeruginosa* is the most common cause of outbreaks in spas and hot tubs and the second leading cause of waterborne diseases from recreational use [2]. Some diseases associated with this bacterium in the water are otitis, conjunctivitis, green nail syndrome, and pneumonia [3–5]. These non-intestinal infections commonly occur through direct exposure of the skin, ear and eye with contaminated water and inhalation of aerosols, especially in susceptible individuals; however, there are no reports of gastrointestinal infection due to ingestion of swimming pool water [6, 7].

International and national guidelines for detection and enumeration of *P. aeruginosa* are used as a basis for monitoring the presence of bacteria in recreational waters and protection of the

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public health against related diseases [7, 8]. However, conventional culture-based methods using the membrane filter (MF) are laborious and time consuming to achieve results [9, 10]. Moreover, regarding exposure to water disinfectants [11], these methods would underestimate the total viable *P. aeruginosa* because of failing to form colonies of viable but non-culturable (VBNC) bacteria in the laboratory media [5, 12]. Changes in physiological and biochemical properties [11] together with the reduced metabolic activity to the baseline levels make VBNC bacteria smaller, non-divisible, and more resistant to antimicrobial factors although these cells remain alive and can regain their pathogenicity under favorable conditions [13]. Therefore, timely detection of VBNC pathogens in relevant environments such as the water and food may be a critical issue in monitoring and prevention of disease outbreaks [14]. To contrast between viable, VBNC, and dead bacteria, some methods have been developed based on viability assays such as respiratory and metabolic activity measurement, cytoplasmic membrane integrity, immunological tests, fluorescent-based hybridization, and molecular methods for DNA and RNA hybridization probes in PCR and reverse transcriptase PCR [6, 12, 15]. Amongst these methods, direct DNA extractions followed by propidium monoazide quantitative polymerase chain reaction (PMA-qPCR) is one of practical methods for assessment of VBNC bacteria in environmental samples [11, 12, 16]. PMA is a DNA intercalating molecule that can differentiate between live and dead or membrane-damaged bacteria. It can selectively penetrate the damaged cells and form a stable covalent high-affinity bonds with DNA, following photo-activation exposure to strong visible light [17]. The DNA-PMA bond inhibits PCR amplification of the DNA strands of dead bacteria and eliminates false results and overestimation of the bacterial count due to extracellular and dead cells DNA in qPCR assays [18]. Therefore, quantification cycle (C_q) values in PMA-qPCR assay will be higher than C_q values in qPCR in the same sample containing dead bacteria [16]. The use of a hybridized probe makes PMA-qPCR a species-specific method to distinct dead/live cells even in environmental bacterial consortiums such as chlorinated swimming pools.

The rising resistance to disinfectants and antibiotics in *P. aeruginosa* strains is another public health concern regarding this opportunistic pathogen in swimming and recreational waters [1, 19]. It is expected that environmental strains of *P. aeruginosa* may be more susceptible to antibiotics. Nevertheless, due to genome complexity, acquired or mutational resistance, and presence of large attached bacterial colonies in some environments, *P. aeruginosa* may become resistant to several classes of antibiotics [20, 21].

To the best of our knowledge, no study has been performed on direct molecular detection and enumeration of VBNC *P. aeruginosa* in swimming pools. Therefore, a PMA pretreatment qPCR method was developed to quantify VBNC *P. aeruginosa* directly from MF samples in natural water-

related microbial matrices and the method was compared with qPCR and traditional culture-based methods.

Materials and methods

Sampling

Water samples (1.0 L) were collected from three different places, including the pool, hot tub, and shower water, of 8 circulating indoor swimming pools (totally 24 samples) in Tehran during periods of maximum bather load, in accordance with the Standard Methods for the Examination of Water and Wastewater [22]. To neutralize the residual chlorine, sodium thiosulfate were added to samples. In addition to the planktonic isolates in water samples, the presence of attached *P. aeruginosa* to the pool wall was investigated using sterile cotton-tipped swabs pre-moistened with Phosphate Buffer Saline (PBS). After removing non-attached bacteria from the walls by spraying saline solution, the likely biofilm/sediments were swabbed ($0.3 \times 20 \text{ cm}^2$). The swabs were transferred to 2.0 mL microtubes containing PBS and were kept in cold storage until laboratory test. Free and total chlorine, temperature, and pH of the samples were recorded onsite, and turbidity was measured in the laboratory.

Quantification of *P. aeruginosa* by culture method

P. aeruginosa detection and enumeration from swimming pools water samples were performed as specified in the Iranian national standard method No. 8869 [23] established based on the international standard ISO 16266:2006 [24]. Briefly, 100 ml water from each sample was filtered through a 0.22- μm pore size, 47-mm diameter nitrocellulose MF (Schleicher & Schuell, UK). The MFs were cultured on selective *P. aeruginosa* M-PA-C agar (Himedia lab, India) and incubated at 42 °C for 24–48 h. The *P. aeruginosa* colonies were enumerated as described by ISO 16266:2006 [24]. Sampled swabs were vortexed vigorously, cultured on M-PA-C agar plates, and incubated as described for MFs. All confirmed positive colonies from water and swab samples were further tested by a combination of standard microbiological phenotypic and biochemical tests [25]. Moreover, species confirmation was performed by qPCR assay using gyrB418F/gyrB490R/gyrB444P primers and probe. The *P. aeruginosa* PAO1 was used as a control standard strain during all microbiological and molecular assays.

Primers and probes for qPCR

A highly species-specific set of primers and probe was previously designed to identify the *gyrB* target gene of *P. aeruginosa*, and analytical specificity was confirmed in 15 non-target species (Table 1) and 10 *P. aeruginosa* strains

Table 1 Bacterial strains used for analytical specificity test of *gyrB* target gene to identification of *Pseudomonas aeruginosa*

Bacterial species	Strain ID	Source
<i>Pseudomonas aeruginosa</i> (PAO1)	ATCC ^a 15,692	Pasteur Institute of Iran
<i>Pseudomonas aeruginosa</i>	ATCC 27833	Pasteur Institute of Iran
<i>Pseudomonas fluorescens</i>	ATCC 13525	IBRC ^b
<i>Pseudomonas stutzeri</i>	IBRC-M 10811	IBRC
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	IBRC-M 10702	IBRC
<i>Pseudomonas putida</i>	ATCC 12633	IBRC
<i>Burkholderia cepacia</i>		M 6 ^c
<i>Stenotrophomonas maltophilia</i>		SMH 85 ^c
<i>Staphylococcus aureus</i>	ATCC 6538	PTCC ^d
<i>Escherichia coli</i>	ATCC 10536	PTCC
<i>Escherichia coli</i>	ATCC 32218	IBRC
<i>Enterococcus hirae</i>	ATCC 10541	PTCC
<i>Bacillus cereus</i>	ATCC 9634	IBRC
<i>Salmonella typhimurium</i>	ATCC 14028	IBRC
<i>Bacillus subtilis</i>	ATCC 6633	IBRC
<i>Acinetobacter baumannii</i>	ATCC 19606	IBRC
<i>Enterococcus faecalis</i>	PTCC 1778	PTCC

^a ATCC: American Type Culture Collection; ^b IBRC: Iranian Biological Resource Center; ^c Clinical strains; ^d PTCC: Persian Type Culture Collection

including two standard, two clinical (CF patients), and eight environmental isolates from Tehran, Iran (data not published). The sensitivity or limit of detection (LOD) of the method was determined using 10-fold serial dilutions of the DNA extracted from *P. aeruginosa* PAO1 with a range from 10^6 to 10^0 genomic equivalents (GE), and the C_q of the last signal of standard determined the LOD of qPCR assays (for cases of no detectable signal for NTC).

The sequences of the forward primer (gyrB418F), reverse primer (gyrB490R), and hydrolysis (TaqMan) probe (gyrB444P) used in qPCR assays were 5'-AACAAAGGTCTGGGAACAGGTCTAC-3', 5'-CATCGG TCTCGCCC ACTTC-3', and 5'-TAMRA-CCACGGCGTTCCGC AGTTCC-BHQ2-3', respectively.

MF Colony and DNA recovery characteristics

In order to compare the results of qPCR and PMA-qPCR assays in a unique sample, we required to know whether the number of colonies on each of MF half was statistically distributed equally or not. Moreover, other objectives of this part of the study were to determine the effect of the type of MF and culture medium on colony formation on the MF, and to determine the analytical sensitivity of the developed method for direct extraction of DNA from MFs in qPCR. One fresh colony of *P. aeruginosa* PAO1 was grown overnight on a shaking incubator (100 rpm) at 37 °C in LB broth; 1 ml of the suspension was inoculated into 9 ml of the fresh LB broth and incubated for 1.5 to 2 h to obtain log-phase cells. Two milliliters of this suspension

was pelleted at 400 x g, re-suspended in sterile PBS, and diluted to an OD₅₉₅ of 0.1. Thirty-two 100 mL aliquots for two concentrations of *P. aeruginosa* ($1.5E3$ and $1.5E2$ cfu/100 mL) were prepared in saline solution and used for determination of random distribution of colonies on MFs, and also for comparing the effect of two brands of MFs and two specific culture media (Cetrimide Agar and M-PA-Agar) on recovery of the colonies. The 100 mL samples were passed through the MF apparatus, and the obtained MFs were cut into two halves and each half was cultured at 37 °C. All cultured MF halves were photographed after 24 and 48 h, and the colonies were counted using the ImageJ software (National Institutes of Health, USA) environment [26]. Moreover, two series of serial 10-fold dilutions of spiked 100 mL samples (10^5 to 10^0 cfu/100 mL) were filtered; the MFs were cut into two equal halves and subjected to DNA extraction. The obtained qPCR results were analysed statistically in order to investigate the distribution of bacteria on MFs and determine the analytical sensitivity of the developed qPCR assay, including MF samples preparation and qPCR analysis.

Sample preparation and PMA treatment for qPCR

PMA (BLU-V Viability PMA Kit; Qiagen, Germany) was dissolved in RNase free water to obtain a 2.5 mM stock solution and stored in refrigerator in dark vials. An optimal concentration of 50 µM PMA was selected from previous studies in order to sample treatment with PMA [18, 27]. To investigate the effect of PMA on live and dead cells in both filtered

and suspended format, the suspensions of *P. aeruginosa* PAO1 were prepared in two groups of 10^6 cfu/100 ml (200 mL samples) and 10^6 cfu/1 ml (1 mL vials), each one in three series (in triplicate) as shown in Fig. 1. First aliquots groups were heat treated in a water bath at 70 °C for 15 min to obtain VBNC bacteria, the second were exposed to 3 mg/L free chlorine for 15 min (dead cells) and the last remained untreated as viable control cells. To evaluate bacterial growth, 10 µL of each aliquot was cultured at 37 °C. The 200 mL samples were filtered and the MFs were cut into two equal halves; One series of the MF halves and 1 mL vials were subjected to PMA treatment, and then exposed to halogen light photoactivation (600 W; Osram, Germany). MF treatment with and without PMA was also done for 200-mL natural water samples of the swimming pool. The PMA-treated and untreated MF halves and vials were subjected to direct DNA extraction as described below.

DNA extraction and qPCR conditions

Each untreated and PMA-treated MF half obtained from spiked samples and the pool water was cut into thin strands (about 1 mm thick) on a sterile plate. These strands were placed into 2.0 mL microtubes containing glass pearl. The DNeasy Blood & Tissue Kit (Qiagen, Germany) was used for direct DNA extraction from MF samples with minor modifications. Briefly, 180 µL Buffer ATL and 20 µL proteinase K (20 mg/mL) were added to microtubes containing MF strands and glass pearl and vortexed for 5 min. Following incubation at 56 °C for 15 min, 200 µL of Buffer AL was added to microtubes and mixed thoroughly through vortexing. The microtubes were sonicated at 35 kHz for 2 minutes and the procedure was done following the kit manufacturer's instructions. The purity of yielded DNA ($OD_{260/280}$) was measured using Epoch microplate spectrophotometer (Biotek Instruments, USA)..

Fig. 1 Preparation of *Pseudomonas aeruginosa* PAO1 in 2 groups (200 mL filtered and 1 mL suspensions samples) and 3 series (heat treated, chlorine treated and untreated cells) to determine the effect of PMA on preventing DNA amplification of damaged cells in qPCR. The image was prepared in Edraw Max v. 9.2

A total volume of 20 μL reaction mix, containing 7.5 μL of the Environmental Master Mix 2.0 (Life Technologies, USA), 0.1 μM of each primer and probe, and 5 μL genomic template DNA was used for qPCR. The thermal cycling condition included an initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and an annealing step at 60 °C for 30 s. Data analysis was performed using the StepOne software v2.3.

Antimicrobial susceptibility testing

Antimicrobial resistance patterns of *P. aeruginosa* isolates from swimming pools were determined against 11 antibiotics using the standard Kirby-Bauer disk-diffusion method (Mast Co., UK) on Mueller–Hinton agar (Merck, Germany). Antimicrobial susceptibility of random colonies of one of the positive water samples was investigated to determine any difference in susceptibility between colonies from the same water source. Since the results of pre-screening tests did not show any difference, we randomly tested only two colonies (isolates) from each positive plate if two or more colonies were available. Totally, ten screened isolates from five positive plates were used in the disc diffusion method and the results were interpreted using Clinical and Laboratory Standards Institute methods [28].

Statistical data analysis

Statistical analysis was performed using the R software version 3.5.0 [29] and the plots were developed using the ‘ggplot2’ and ‘dplyr’ package. The normal distribution of the colony counting data was investigated using the Shapiro-Wilk normality test. The homogeneity of the variance in the mean number of colonies on different media ($n = 2$) and MFs ($n = 2$) was tested using the Fligner-Killeen test (P value > 0.05). ANOVA were used to compare the mean number of colonies on different media and MFs. The post-ANOVA Tukey HSD test was applied to determine the difference between means ($\alpha = 0.05$). In addition, parametric and nonparametric t-tests were used to compare the GE/ C_q results of PMA-qPCR optimization and swimming pool samples before and after PMA treatment, respectively. Moreover, the 90% percentile of the culture-based and qPCR method results was used to compare the efficacy of methods for detection of *P. aeruginosa* in swimming pool samples. The tests were biologically and technically repeated at least twice, unless otherwise stated.

The two-way single score intra-class correlation coefficient (ICC) (95% CI) was calculated for test–retest reliability and assessing agreement between two MF halves series using the ‘irr’ package [30], and results equal to or greater than 0.8 were considered acceptable. The internal consistency between two

series of half MFs was investigated by Cronbach’s alpha using the ‘rela’ package [31].

Results and discussion

Analytical specificity and sensitivity of developed qPCR assays

The selected gyrB418F/gyrB490R/gyrB444P primers and probe used for detection of *P. aeruginosa* in the study yielded negative results in all 15 non-target species (Table 1), while they amplified the target sequence of all 12 *P. aeruginosa* target isolates/strains. The qPCR amplified the target *gyrB* gene of *P. aeruginosa* PAO1 at a 10-fold serial dilution from 10^6 to 10 GE in all replicates with a linear and robust standard curve. Therefore, the sensitivity (LOD) of qPCR assay was determined as 10 GE (equivalent to 9.6 cfu/100 mL), with no detection signal for negative controls and an amplification efficiency of 107% (R^2 0.99) as shown in Fig. 2.

Detection of *P. aeruginosa* by standard culture-based and qPCR methods

Totally, 24 water samples and 16 swab samples were collected and analysed from eight public indoor swimming pools in the south and centre of Tehran, Iran. Out of 24 water samples and 16 swab samples from 8 swimming pools, 6 water samples (25%) from 5 and 2 swab samples from 2 swimming pools were contaminated with *P. aeruginosa*, as confirmed by the standard culture-based method, and one of positive samples was considered negative as confirmed by biochemical and qPCR tests. Contamination density ranged from 1 to 7 cfu/100 mL. The culture-based results of this study were in accordance with studies conducted in other Iranian cities. About 26%, 13%, and 21% of the samples taken from Shiraz [32], Isfahan [33], and Yazd [34] indoor swimming pools were positive for *P. aeruginosa*, respectively. However, a study conducted in Tehran reported a higher contamination rate of about 82% for positive samples [1].

All samples were also analysed using the qPCR method. The positivity rate for *P. aeruginosa* by qPCR was 18 (75%) in 8 swimming pools, ranging from 27 to 2090 GE (Table 2).

As shown in Table 2, although one of the samples (F2) had positive results in the culture-based method, further biochemical and qPCR tests rejected the positivity of this sample. This finding can be due to the non-specific nature of the M-PA-C agar medium or the high phenotypic similarity of other species of the *Pseudomonas* genus to *P. aeruginosa*. However, standard culture-based methods for detection and enumeration of *P. aeruginosa* in water samples may have false-positive results [35]. Furthermore, the trustworthiness of other laboratory methods such as the API20NE identification system is under

Fig. 2 Standard curve, sensitivity and efficiency of qPCR assay for 10-fold serial dilution (10^6 –1 GE) of the *Pseudomonas aeruginosa* PAO1; $C_q = -3.147 \log_{10}(\text{GE}) + 40.32$

question, because they have been developed based on pathogenic species identification and therefore they often fails to identify environmental isolates [36]. This limitation, besides the long time (up to 6 days) to achieve results in culture methods [37], makes qPCR methods an appropriate alternative to identify *P. aeruginosa* in environmental samples [38].

In order to enumerate the bacteria in diluted environments such as treated water in which the bacterial count is low, it is necessary to concentrate the high volume of the samples by filtering it using the MF. Although plating MFs is a simple process in the standard culture method, complete bacterial isolation from an MF is a limitation in qPCR assays. Therefore, developing a method for direct extraction of the bacterial DNA from the MF is one of the critical steps in

sampling aquatic environments to detect pathogens by qPCR methods. Comparison of culture-based and qPCR results in Table 2 emphasizes the efficiency of direct DNA extraction from MFs.

Statistical model of Colony and DNA distribution on MFs and recovery effect

The statistical results of the colony count on each of the two series of MF halves obtained from 32 samples and C_q obtained from 12 samples demonstrated that the filtered bacteria were randomly distributed on the MFs with a normal distribution (P value = 0.98). Moreover, variations between each two series of MF halves, in terms of bacterial count or bacterial GE, was

Table 2 Comparison of results obtained by culture-based, qPCR, and PMA-qPCR (viable) methods for enumeration of *Pseudomonas aeruginosa* in Tehran indoor swimming pools

Swimming pool code	Sample code	Sample type	Final count by reference culture (cfu/11 mL) method	Bio-Chemical confirmed colonies	qPCR confirmed colonies	qPCR (GE/100 mL)	PMA-qPCR (GE/10 mL)	Presence of VBN <i>P. aeruginosa</i>
A	A1	Shallow Pool	0	NT ^a	NT	1032 ± 112	161 ± 1	+
	A3	Semi-deep Pool	0	NT	NT	<LOD ^b	<LOD	–
	A4	Shower water	7	+	+	98 ± 8	76 ± 3	+
	B2	Shallow Pool	0	NT	NT	81	<LOD	–
B	B3	Hot tub	0	NT	NT	<LOD	<LOD	–
	B4	Shower water	4	+	+	106 ± 31	96 ± 34	+
	C1	Deep Pool	7	+	+	65 ± 7	60	+
	C2	Shallow Pool	1	+	+	<LOD	<LOD	–
C	C3	Hot tub	0	NT	NT	668 ± 2	<LOD	–
	D1	Semi-deep Pool	0	NT	NT	27 ± 3	31 ± 3	–
	D2	Hot tub	0	NT	NT	<LOD	<LOD	–
	D3	Shower water	0	NT	NT	46 ± 30	<LOD	–
E	E1	Semi-deep Pool	0	NT	NT	<LOD	<LOD	–
	E2	Hot tub	0	NT	NT	33	<LOD	–
	E3	Shower water	0	NT	NT	33	<LOD	–
	F1	Semi-deep Pool	0	NT	NT	372 ± 137	248 ± 31	+
F	F2	Hot tub	3	–	–	134	<LOD	–
	F3	Shower water	0	NT	NT	95	<LOD	–
	G1	Semi-deep Pool	0	NT	NT	949	<LOD	–
	G2	Hot tub	0	NT	NT	<LOD	<LOD	–
G	G3	Shower water	0	NT	NT	292 ± 76	50 ± 24	+
	H1	Semi-deep Pool	0	NT	NT	228	80	+
	H2	Hot tub	0	NT	NT	2090 ± 259	1350 ± 91	+
	H3	Shower water	1	+	+	176 ± 27	143 ± 9	+

^a NT: not tested; ^b < LOD: The GE/100 mL was lower than 10

not statistically significant (P value = 0.416). Also, the calculated ICC was greater than 0.8, and therefore, there was an excellent agreement between every two series of MF halves. Since the bacterial distribution on the MF followed a random and normal distribution, it would be applicable to use the results of bacterial measurement in parallel for both halves of the same MF to compare culture-based, qPCR and PMA-qPCR methods.

There were no statistical differences between the results of colony recovery from two tested culture media; however, the MF type had a significant effect on colony recovery at a concentration of 1.5×10^3 and the average of the recovered colonies in the MF brand A was significantly higher compared to brand B. The efficiency of the qPCR assay for direct DNA recovery from MFs was investigated using 10-fold dilutions (10^5 to 10^0 cfu/100 mL) and compared with the related count of colonies obtained by the aliquot and MF plating (data not shown). However, the analytical sensitivity of the qPCR assay (including sample preparation, extraction and qPCR) was determined to be 10^1 GE.

Effect of PMA treatment on C_q of viable, heat-treated and chlorine-Treated *P. aeruginosa*

The signal reduction capacity of PMA-treated MFs was assessed by comparing PMA-qPCR and qPCR assays for viable, VBNC and dead *P. aeruginosa*. Heat and chlorine treatment resulted in complete loss of *P. aeruginosa* culturability, as confirmed by plating a 10- μ L aliquot from each suspension. When the C_q differences (ΔC_q) of PMA-treated and untreated MF samples were compared, a maximal signal shift of about 4.5 and 13.5 to higher C_q values signals was observed for heat-treated and chlorine-treated *P. aeruginosa* PAO1 respectively, while this ΔC_q was lower than 1 for viable (untreated) samples. The ΔC_q (C_q differences before and after PMA process) of heat-treated and chlorine-treated *P. aeruginosa* were statistically significant and correlated with the defined range of 6 ± 2 for ΔC_q of the damaged cells before and after treatment with PMA, as suggested by the kit manufacturer. The relatively high ΔC_q of heat treated samples before and after PMA-treatment may be caused by partial damage to viable cells due to heat treatment. Therefore, it can be deduced that PMA treatment in described optimization can

prevent qPCR amplification of DNA from damaged cells and cell-free DNA in water samples, while it has no significant effects on the viable and VBNC bacteria, which results in less overestimation of the bacteria by qPCR due to well discrimination between viable and dead *P. aeruginosa*. With a lower cytotoxic effect on intact bacteria in comparison to other molecular dyes such as ethidium monoazide (EMA), the probability of false negative results for PMA is negligible [39, 40], while the detection power of PMA-qPCR is much higher than the culture method. In addition, direct application of PMA to the MF, besides strong inhibition of DNA amplification, eliminates the further processes for detachment and resuspension of the bacteria from MFs that may result in missing the bacteria in low-count water samples.

Cross comparison of qPCR and PMA-qPCR methods for the determination of VBNC *P. aeruginosa* in natural samples

To evaluate the usefulness of the developed PMA-qPCR method for detection and enumeration of viable and VBNC *P. aeruginosa* in aquatic environments, 24 filtered water samples taken from swimming pools were tested simultaneously by the standard culture method, conventional qPCR, and PMA-qPCR (Table 2). Due to the different nature of the culture-based and qPCR methods, we were not able to calculate the pure numerical values for VBNC (cfu vs. GE unit), but with numerical comparison of the results of qPCR before and after PMA treatment with standard culture results, we were able to determine the presence of VBNC bacteria. As seen in Table 2, we have 3 critical column for results entitled final count by reference culture (cfu/100 mL), qPCR (GE/100 mL) and PMA-qPCR (GE/100 mL) that are related to “cultureable”, “dead, viable and VBNC” and “only viable and VBNC” *p. aeruginosa* respectively. Although the cfu and GE are different numerical unit and cannot be combined in an mathematical equation, but knowing that each bacterium has only one genome, so, each GE is exactly equal to one cfu for viable and VBNC bacteria. Therefore, according to the numerical data of the three columns we have given, we can distinguish between viable and VBNC bacteria. If we consider the unique nature for cfu and GE, then this equation may be

Table 3 Comparison of positive results of standards microbiological, qPCR, PMA-qPCR and VBNC presence from 24 swimming pool samples separated by the sampling point

Swimming pool sampling point	Reference culture-based method	qPCR method	PMA-qPCR method	VBNC bacteria presence
Main pool water	2/10 (20%)	7/10 (70%)	5/10 (50%)	4/10 (40%)
Hot tub water	0/7 (0%)	4/7 (57%)	1/7 (14%)	1/7 (14%)
Shower water	3/7 (43%)	7/7 (100%)	4/7 (57%)	4/7 (57%)
Total water samples	5/24 (21%)	18/24 (75%)	10/24 (42%)	9/24 (37.5%)

applicable for VBNC calculation:

VBNC *P. aeruginosa* = PMA-qPCR–cultured colony number

We proceeded with caution in this calculation and in the case of VBNC; we only mentioned the presence and absence (positive and negative) bacteria in Table 2.

The results of qPCR and PMA-qPCR were compared using the Wilcoxon signed rank test analysis and significant differences were seen in GE results before and after PMA treatment as expected (P value = 0.00038). A mean decrease of 2.21 log (163 GE) was observed after treatment of MFs with PMA. The positive results of samples taken from the swimming pool water tested for the presence of *P. aeruginosa* using the three methods are compared in Table 3. None of the samples taken from seven hot tubs were positive in the culture-based method, and only one of them were positive for VBNC *P. aeruginosa*. This results may be due to the high level of free chlorine and high temperature in hot tubs as measured during sampling (data not shown). On the contrary, the half of shower samples were positive for culture and VBNC, because the level of free and total chlorine in the water of all showers was zero. The high level of VBNC positive samples may be due to the effect of temperature on stimulating the bacteria to change their state to the VBNC mode. Also, this high positivity rate for shower samples may probably result from biofilm formation on the head, tap, and pipe connections of the showering system. It is proved that high temperature and disinfectant concentration can stimulate *P. aeruginosa* to shift to the VBNC mode as a resistant bacterial form [41]. However, the 90% percentiles of the results for culture-based and PMA-qPCR (viable bacteria) were 3.7 cfu/100 mL and 718 GE/100 mL that support the efficacy of molecular methods for detection of *P. aeruginosa* in environmental samples. Moreover, there was about 17% difference between positive results of culture-based and developed VBNC method in all samples, which makes qPCR assays along with PMA treatment a promising, rapid and relatively inexpensive alternative for detection and enumeration of *P. aeruginosa* in water samples.

Susceptibility of *P. aeruginosa* isolates to antimicrobial discs

Totally, 10 *P. aeruginosa* colonies were isolated from four swimming pools (the isolate from a pool was not confirmed by biochemical and qPCR) were subjected to susceptibility testing. Except for isolates 2 and 7 that were intermediately resistant to Aztreonam and Ticarcillin/clavulanic acid respectively, the remaining isolates were susceptible to all 11 antibiotics. The antibiotic resistance of environmental isolates such as water samples may be less than clinical isolates [5]. In contrary, environmental isolates such as swimming pool strains are more resistant to antimicrobial disinfectants including chlorine [4, 42], especially non-human sources of *P. aeruginosa*. Another reason for the

low rate of antibiotic resistant *P. aeruginosa* in our study may be the study design because only recreational pools were sampled and no sampling was done in therapeutic pools.

Conclusion

The present study shows that the selected gyrB418F/gyrB490R/gyrB444P as a high species-specific and sensitive set of primers and hydrolysis probe for molecular identification of *P. aeruginosa*, is a useful, valid and rapid alternative to conventional plate count methods. In this study, it is demonstrated that PMA treatment can be used to avoid the false positive results in qPCR through reduce the signal of damaged bacteria in filtered water samples. The developed PMA-qPCR method has no significant effect on viable and VBNC bacteria; thus, PMA-qPCR can detect the VBNC bacteria directly from aquatic samples, which helps to detect and prevent of pathogen outbreaks from water environments and facilitates the evaluation of health risks and quantitative microbial risk assessment. However, there is a limitation: the PMA-qPCR method can detect 10 GE of *P. aeruginosa* directly from MF samples. Nevertheless, the PMA-qPCR method is not affected by environmental confounder and can be used with accuracy in chlorinated recreational water samples. Other findings from this study are the random distribution of bacteria and effect of MF on the colony formation on the MF. Therefore, with precise selection of MF, equal parts of it can be used simultaneously to compare two or more test methods, such as qPCR/PMA-qPCR.

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Compliance with ethical standards

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

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