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Detection of *Pseudomonas aeruginosa* by a triplex polymerase chain reaction assay based on *lasI/R* and *gyrB* genes

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Abstract *Pseudomonas aeruginosa* is a nosocomial pathogen, which, due to its inherent and acquired resistance to a wide range of antibiotics, causes high mortality rates. Therefore, rapid detection of the bacterium with high specificity and sensitivity plays a critical role in the control of the pathogenic bacterium. The aim of this study was to evaluate the accuracy and specificity of a prompt detection of the bacterium based on a triplex polymerase chain reaction that amplifies the *lasI*, *lasR* and *gyrB* genes.

For this purpose, 30 clinical isolates of *P. aeruginosa* and 30 wound biopsy samples were retrieved from clinical diagnostic laboratories. After the extraction of the chromosomal DNA, the desired genes were amplified using uniplex and triplex PCR with appropriate primers. The specificity of the primers was evaluated by a comparison of the PCR results for *P. aeruginosa* clinical samples and non-*Pseudomonas* species control samples. The sensitivity of the primers was determined using a serial dilution of the genomic DNA template (100 ng to 100 fg) and by a comparison of the PCR and bacterial culture results.

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The results showed that the triplex PCR assay was positive for all of the samples (100%), while the PCR identifications were negative for non-*Pseudomonas* species. Additionally, at 10^{-4} and 10^{-5} diluted genomic DNA from *P. aeruginosa* (10 pg and 1 pg), the triplex PCR test was positive for the *Las* and *gyrB* genes in all of the samples, respectively. Based on these results, the designed primers can be used for the rapid, specific and sensitive diagnosis of *P. aeruginosa* in a triplex PCR assay.

Introduction

Pseudomonas aeruginosa is one of the most important and opportunistic pathogens that causes a high rate of mortality and morbidity in hospitalized patients with compromised immune systems [1,2]. *Pseudomonas* infections are generally treated by antibiotics; however, unfortunately, in hospitalized patients, these infections are becoming more difficult to be treated, specifically because of the increasing number of antibiotic-resistant strains. In recent years, infections caused by this bacterium are one of the major problems in hospitals and are associated with high rates of mortality, which range from 18% to 61% [3—7]. Thus, early diagnosis and proper medical treatments are the best strategies for fighting against these infections [8]. In most laboratories, the detection of *P. aeruginosa* is still accomplished by microbiological culture and biochemical tests. Although a comparative study has shown that these methods contain reliable detection results, they are time-consuming and require several days to be completed [9,10]. Studies have shown that inappropriate initial antimicrobial therapies are associated with adverse outcomes for infection treatments. Conversely, false detection can result in the administration of ineffective antimicrobial therapies during the first 48—72 h [11,12]. Moreover, in some cases in which the bacterial count is low, especially in antibiotic-treated patients, false-negative results can be achieved in routine laboratory tests. Thus, access to rapid and specific methods that have a high sensitivity is of a great importance. In recent decades, the detection and identification of *P. aeruginosa* in clinical samples by polymerase chain reaction (PCR) has been increased substantially [13,14]. Since 1992, when McIntosh et al. reported PCR detection of *P. aeruginosa* for the first time, multiple genes have been reported as PCR targets for the identification of this bacterium [13,15—18]. Afterward, various studies have shown that these genes do not have complete sensitivity or specificity for bacterial detection and, thus, have false-negative and false-positive results. It should be noted that *P. aeruginosa*'s genome has a highly polymorphic nature, which can influence the reliability and specificity of the PCR. Therefore, the use of a single gene target could lead to unexpected errors, including cross reactions with other bacterial species and false-negative or false-positive results, and a highly stringent and distinctive PCR assay is needed [19,20]. In this study, we have developed and validated a triplex PCR assay for the detection of *P. aeruginosa* using three different genes, including the *lasI*, *lasR*, and *gyrB* genes. It has been reported that the *gyrB* gene, which encodes the subunit B of DNA gyrase, is a reliable PCR target for *P. aeruginosa* detection [21], while the *lasI* and *lasR* genes are essential quorum sensing (QS) genes of the bacterium. According to studies, QS is necessary for the development of infection by *P. aeruginosa*, and the QS genes are exclusive and conserved for each bacterial species [22—25]. Thus, given the importance of these genes in the pathogenicity of *P. aeruginosa*, *las* genes can be used for the detection of *P. aeruginosa* in the PCR assay. Because some studies have shown few clinical isolates with quorum sensing-deficient systems [26], we include the *gyrB* gene to reduce false-negative results in the detection process.

Materials and methods

Bacterial strains

Thirty clinical isolates of *P. aeruginosa* and thirty wound biopsy samples were retrieved from clinical diagnostic laboratories in Khatam-al-Anbia and Shahid Motahari Hospitals (Tehran, Iran). All of these isolates were identified by conventional biochemical and microscopic methods. *P. aeruginosa* (ATCC 27853) and non-*P. aeruginosa* species, including *Pseudomonas fluorescens* (ATCC

Table 1 Primer sequences.

Target gene	Nucleotide sequence	Tm	Amplicon size (bp)
lasI-F	5′-ATGATCGTACAAATTGGTCGG-3′	66 ºC	600
lasI-R	5′- GTCATGAAACCGCCAGTC-3′	67 ºC	
lasR-F	5′-ATGGCCTTGGTTGACGGT-3′	65 ºC	700
lasR-R	5′-GCAAGATCAGAGAGTAATAAGACCC-3′	66 ºC	
*gyr*B-F	5′-CCTGACCATCCGTCGCCACAAC-3′	65 ºC	222
*gyr*B-R	5′-CGCAGCAGGATGCCGACGCC-3′	68 ºC	

17386), *Pseudomonas syringae* (CCM 2868), *Pseudomonas pertucinogena* (ATCC 190), *Pseudomonas putida* (ATCC 12633) and *Burkholderia* (*Pseudomonas*) *cepacia*, were received from the Iranian Biological Resource Center (IBRC). *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella enteric* serovar *Typhimurium* clinical isolates (also received from Khatam-al-Anbia and Shahid Motahari Hospitals) were used as controls for the cross-reaction analyses.

DNA extraction

Genomic DNA was extracted using a DNA Pure Extraction Kit (Bioneer, South Korea) according to the manufacturer's recommendations. Genomic DNA of all of the clinical samples was also directly extracted using MolYsis kit (Molzym, Germany). The amount and purity of the extracted DNA was measured using a Nanodrop ND1000 spectrophotometer (Thermo, USA).

Primer design

The sequences of the *lasI*, *lasR* genes were adopted from GenBank accession number NC_002516.2. Forward and reverse primers (Table 1) were designed using Oligo and DNAsis software. The *las* primers were evaluated using the BLAST service to ensure their specificity for *P. aeruginosa*. Specific primers for the *gyrB* gene were the same as in Qin et al. [27].

PCR amplification with uniplex and triplex assays

The PCR reaction for the amplification of each gene was performed as follows. Fifty microliter reactions were prepared. Each reaction contained 5 μl of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Ampliqon, Denmark), 20 pmol of each primer, 1 unit of Taq DNA polymerase (Ampliqon, Denmark), and 100 ng of DNA template. The reaction cycled through the following temperature profile: an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s. Finally, the samples were subjected to 72 °C for 5 min for final extension. The PCR products were analyzed using %1 agarose gel electrophoresis. GeneRuler 100 bp DNA Ladder (Thermo, USA) was used to determine the size of the amplified fragments. Triplex PCR was performed by combining the primers of all three genes in the same PCR reaction.

Determination of PCR specificity

To determine the specificity of the designed primers in uniplex and triplex PCR assays, genomic DNA from *S. aureus*, *K. pneumoniae*, *E. coli*, *S. typhimurium* and non-*P. aeruginosa* species were used as templates. PCR reactions using *lasI*, *lasR* and *gyrB* gene-specific primers were conducted under reaction conditions, as stated before.

Determination of PCR sensitivity

After preparation of a given concentration of genomic DNA (100 ng/μl), a serial dilution of samples was prepared from 10^{-1} to 10^{-6} concentrations using sterile distilled water. PCR reactions were performed according to the previously mentioned conditions. PCR test sensitivity in some studies is evaluated based on the similarity between the results obtained from PCR assay and standard methods. In addition, the accuracy can be obtained based on the PCR results from the lowest DNA template dilution [13]. Alternatively, the sensitivity of PCR could be determined based on the bacterial DNA copy numbers. For this purpose, we used the formula presented in the URI Genomics and Sequencing Center by Andrew Staroscik in 2004.

$$\text{Number of copies} = \frac{\text{DNA amount (ng)} \times 6.022 \times 10^{23}}{\text{length (bp)} \times 1 \times 10^{9}\ \text{(ng/g)} \times 650\ \text{(g/mole of bp)}}$$

Statistical analysis

The statistical measures of sensitivity and specificity were calculated through a comparison of the bacterial culture (as the gold standard method) and our new approach. To confirm the accuracy of the method, the PCR was performed three times for each sample.

Results

PCR of *lasI*, *lasR*, and *gyrB* genes

The *lasI*, *lasR*, and *gyrB* genes were amplified from the genomic DNA of standard *P. aeruginosa*, clinical isolates and biopsy samples by uniplex and triplex PCR. The produced amplicons were 600 bp, 700 bp, and 222 bp for the *lasI*, *lasR*, and *gyrB* genes, respectively. For all of the samples of *P. aeruginosa*, the PCR results were positive (Figs. 1 and 2).

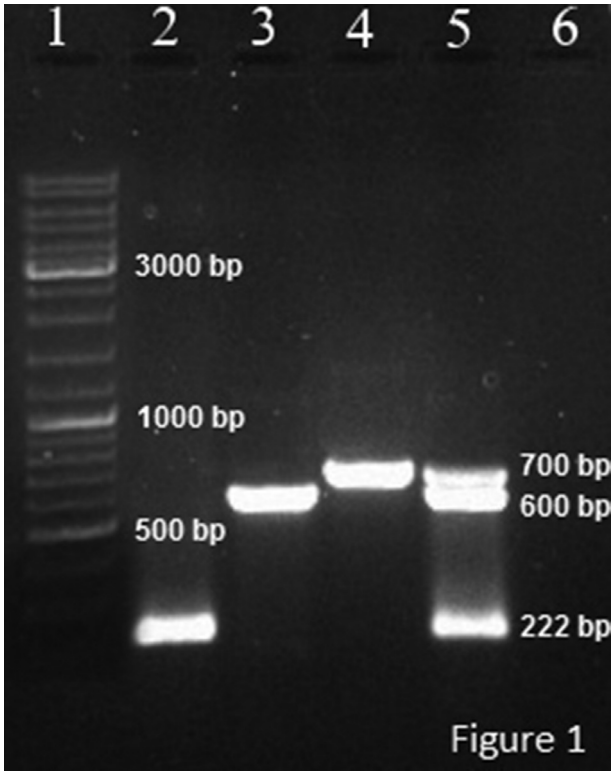

Figure 1 Uniplex and triplex PCR of *P. aeruginosa*. Lane 1: GeneRuler 100 bp DNA Ladder; Lane 2: *gyrB* amplicon (222 bp); Lane3: *lasI* amplicon (600 bp); Lane 4: *lasR* amplicon (700 bp); Lane 5: triplex PCR for *gyrB*, *lasI* and *lasR* genes of *P. aeruginosa* ATCC 27853; Lane 6: triplex PCR without template.

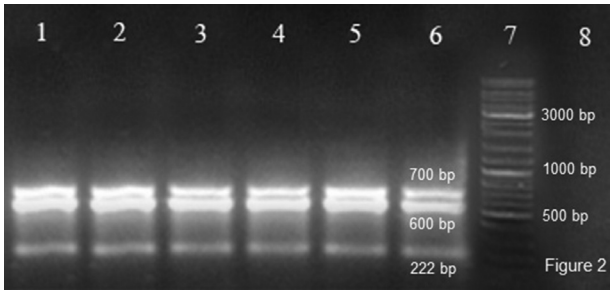

Figure 2 Triplex PCR of clinical *P. aeruginosa* isolates. Lane 1—3: triplex PCR of *gyrB*, *lasI* and *lasR* by DNA of clinical isolates extracted from bacterial culture; Lane 3—6: triplex PCR of *gyrB*, *lasI* and *lasR* by DNA of wound biopsy samples extracted using Kit; Lane 7: GeneRuler 100 bp DNA Ladder; Lane 8: PCR without template.

PCR specificity for the *lasI*, *lasR*, and *gyrB* genes

The genomic DNA of the *S. aureus*, *K. pneumoniae*, *E. coli*, *S. typhimurium* and non-*P. aeruginosa* species were used as DNA templates to obtain the specificity of the primers in PCR assays. The results showed that the designed primers were specific to *P. aeruginosa* genes and that no amplicons were generated when the genome of another species was used (Table 2).

PCR sensitivity for *lasI*, *lasR*, and *gyrB* genes

Because the infectious dose of bacteria is the main parameter in bacterial infections, it is important that the PCR assays be sensitive to the lowest number of this bacterium. Therefore, the genomic DNA of *P. aeruginosa* was extracted, and a serial dilution from 100 ng to 100 fg/μl was prepared. Subsequently, uniplex PCR was performed for each concentration. In uniplex PCR, 10, 10 and 1 pg of diluted DNA were the minimum concentrations of genomic DNA that were detected for the *lasI*, *lasR* and *gyrB* genes amplicons, respectively (Fig. 3A—C). Additionally, in triplex PCR, the minimum concentration of detectable DNA was 10 pg (Fig. 3D). The sensitivity of the PCR was determined based on the bacterial DNA copy numbers. In this study, according to the minimum concentration of the DNA that can be visualized on the gel and the length of the bacterial genome (6264×10^3 kbp), the detectable copy number of bacterial DNA by the *lasI*, *lasR* and *gyrB* genes in uniplex PCR was 1000, 1000, and 100, respectively, and in triplex PCR it was 1000.

Table 2 Determination of PCR specificity using genomic DNA of non-*P. aeruginosa* species and other bacteria as PCR templates.

Bacterial Species	PCR Amplification*		
	lasI	*lasR*	*gyrB*
Pseudomonas aeruginosa	+	+	+
Pseudomonas fluorescens	-	-	-
Pseudomonas pertucinogena	-	-	-
Pseudomonas syringae	-	-	-
Burkholderia cepacia	-	-	-
pseudomonas putida	-	-	-
Staphylococcus aureus	-	-	-
Klebsiella pneumoniae	-	-	-
Escherichia coli	-	-	-
Salmonella typhimurium	-	-	-

[a] The data in each column represent three independent experiments.

Statistical analysis

All of the positive and negative samples in the culture test had the same results in the PCR detection procedure; therefore, the specificity and sensitivity of the detection of *P. aeruginosa* based on *lasI*, *lasR* and *gyrB* gene amplification is 100%. The results obtained from repeating the steps did not show any differences, and thus, *lasI*, *lasR* and *gyrB* gene amplification possesses a high accuracy in *P. aeruginosa* detection.

Discussion

P. aeruginosa is one of the most extensive and serious factors in nosocomial infection. Van der Waaij has shown that 10 to 100 cells of *P. aeruginosa* can lead to gut colonization in immune-suppressed patients who are in intensive care units [28]. Additionally, Ohman et al. and Hazlett et al. have reported that 10^4 cells of *P. aeruginosa* per ml can cause ocular infection in mice, and therefore, early detection of this bacterium is critical [29,30]. Various methods have been developed for rapid and accurate identification of *P. aeruginosa*. Among these methods, PCR, in comparison to the other methods, has a lower cost and is highly accurate and specific.

In 1992, for the first time, the detection of *P. aeruginosa* via the PCR method was reported by McIntosh et al. They used the *algD* gene, which encodes GDP mannose dehydrogenase, a major enzyme in biosynthesis of alginate by *P. aeruginosa* [16]. They reported that *P. aeruginosa* could be specifically detected with a precision of approximately 10 bacterial cells in sputum. In this study, they used a nested PCR method with two pairs of primers, but BLAST analysis of two primers (Pa1 and Pa2) showed 88% and 100% sequence identity with the *algD* gene of *Azotobacter vinelandii*. Studies have shown that the *algD* gene in this bacterium has a high degree of identity (79%) at the DNA level with the *algD* gene from *P. aeruginosa* [31]. Khan and Cerniglia, in 1994, also used a PCR method, which was based on targeting and amplifying a 396-bp region of the exotoxin A (*ETA*) gene sequence [9]. The precision of the PCR assay was reported to be 100% and 96%, respectively. According to their report, by targeting *ETA*, as few as 5—10 cells in 10-ml water samples or 0.1 pg of DNA per reaction mixture (5 μl) could be detected. In 1997, De Vos et al. evaluated the detection of *P. aeruginosa* by triplex PCR using primers that were designed based on two outer membrane lipoprotein genes, *oprI* and *oprL*. In this study, two amplicons, which were produced by the *oprI* and *oprL* genes, were observed only in *P. aeruginosa* isolates, while only the *oprI* gene was amplified from the other *pseudomonas* species. Likewise, the PCR results for all of the other bacteria were negative. The lowest detection level for *P. aeruginosa* was estimated to be 10^2 cells/ml [12]. On the other hand, similar to McIntosh et al., Da Silva Filho and colleague [32] also evaluated the identification of *P. aeruginosa* in clinical samples that were obtained from patients

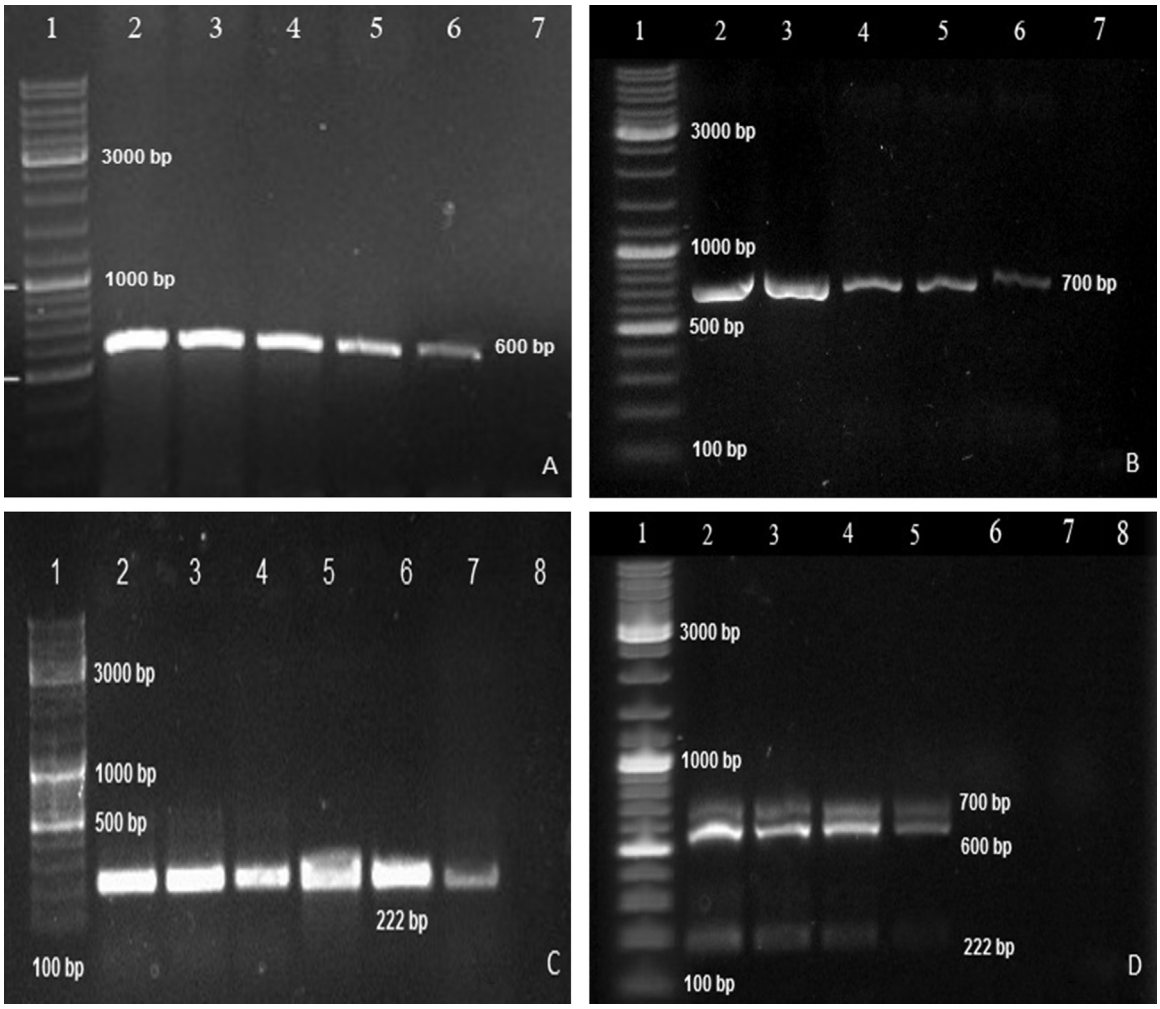

Figure 3 Sensitivity analysis of designed primers in uniplex (A: by *lasI* primers, B: by *lasR* primers, C: by *gyrB* primers) and triplex PCR of *P. aeruginosa* (D). Lane 1: 100 bp ladder; Lane 2: non-diluted template; Lane 3: 1/10 dilution; Lane 4: 1/100 dilution; Lane 5: 1/1000 dilution; Lane 6: 1/10,000 dilution; Lane 7: 1/100,000 dilution; and Lane 8 (C): 1/1,000,000 dilution. For triplex PCR, the first dilution is 1/10, which means that it has 10 ng of DNA template.

with cystic fibrosis by PCR using primers for the *algD* gene. In this study, the specificities of the primers were as high as 100%, and the method could detect 100 pg of bacterial DNA (good sensitivity). However, in comparison to new primers in our study, they show less sensitivity (10 pg of bacterial DNA for our primers). It should be noted that based on BLAST analysis, the reverse primer in their study is 100% identical to a part of the genome of *Pseudomonas putida*, and thus, it is not specific for *P. aeruginosa*.

From 2000 to 2011, many studies have been developed to provide appropriate genes for the identification of *P. aeruginosa* using specific primers with high sensitivity and specificity (Table 3). According to these studies, targeting *gyrB*, *toxA* and transcribed 16S—23S rDNA internal transcribed spacer (ITS) genes in PCR assays can dissociate *P. aeruginosa* from other species with 100% specificity while other genes, including 16S rDNA, *oprL*, *oprI* and *fliC*, have lower specificity. Unspecific amplification was detected with 16SrDNA primers for *Pseudomonas fragi*, *oprL* primers for *Pseudomonas balearica*, and *Pseudomonas citronellolis*, *oprI* primers for *Pseudomonas viridiflava*, *P. balearica*, and *P. citronellolis*, and *filC* primers for all non-*P. aeruginosa* species, except for *P. fluorescens* and *P. citronellolis* [13,21,33]. These false-positive PCR results show a high sequence conservation of these genes among *pseudomonas* species, which can cause inoperative gene targeting for the detection of *P. aeruginosa* via PCR. On the other hand, Lavenir et al. [13] have shown that the PCR assay using the *toxA* gene has PCR products for all of the *P. aeruginosa* samples (100% specificity); however, similar to the Khan and Cerniglia results, the

Table 3 Genes that were used for *P. aeruginosa* detection by uniplex and triplex PCR in previous studies.

Genes	*Production*	*Reference*
algD	GDP-mannose dehydrogenase	(McIntosh et al 992)
16S rDNA	Ribosomal DNA sequence	(O'Callaghan et al. 1994)
toxA	Exotoxin A precursor	(Khan and Cerniglia, 1994)
16s-23s rDNA Internal Transcribed Spacer		(Tyler et al. 1995)
fliC	C-terminus flagellin	(Spangenberg et al. 1996)
oprI&oprL	Lipoprotein I& outer-membrane peptidoglycan-associated lipoprotein	(De Vos et al. 1997)
gyrB	DNA gyrase subunit B	(Qin et al. 2003)
ecfX	Extracytoplasmic function (ECF) sigma factors	(Lavenir et al, 2007)

sensitivity was 95%. According to these studies, targeting of the *toxA* gene for the identification of *P. aeruginosa* has an error of approximately 5%, which can indicate its diversity. Because of the limitations in the phenotypic methodologies, including the biochemical tests, thus far, there has not been a single test that could reliably identify *P. aeruginosa*. Existing molecular assays also have specificity problems, which arise from the horizontal transfer of *P. aeruginosa* genetic material to other *Enterobacteriaceae* species and variations in the genomic DNA [19]. According to these studies, to decrease the specificity problems in the identification of *P. aeruginosa* by PCR, the triplex assay is highly suitable because a multi-target system evaluates more than one factor simultaneously, which could lead to the elimination of false-positive and negative results [12,19]. It is noteworthy that the early detection of the bacterium using specific genes plays a key role in the control of the infection. Considering these points and given that the main virulence factors that cause the pathogenicity of *P. aeruginosa* are controlled by the quorum sensing system genes, these genes are appropriate candidate targets in PCR assays. On the other hand, several systematic studies on *P. aeruginosa* by transcriptional profiling experiments using microarrays have demonstrated that QS is a global regulatory network that controls the expression of over 300 genes by QS inducers [34]. Although the 16S ribosomal RNA (rRNA) gene is the most commonly used gene for genetic identification and characterization of these bacteria, studies have shown that the sequences of *gyrB* gene of *P. aeruginosa* and other species of *Pseudomonas* revealed a higher divergence than 16S rRNA genes. The reason is that the *gyrB* gene rarely transmitted horizontally and its molecular evolution rate is higher than that of 16S rRNA [21,35]. Therefore, the *gyrB* gene is a more appropriate candidate target than 16S rRNA for the identification of the pseudomonas species. However, BLAST results for *gyrB* primers showed that the *gyrB* primer sequences designed by Qin et al. [27] are 100% identical to the *gyrB* gene from *Pseudomonas composti*. This bacterium is a novel species that was reported in 2011 by Gibello [36]. The *gyrB* gene fragment of this strain has a 99% identity to *P. aeruginosa*, and thus, it is possible to obtain false-positive results with *gyrB* uniplex PCR. Moreover, BLAST results showed that designed primers for the *lasI* and *lasR* genes have a complimentary region in all of the *P. aeruginosa* strains and, in addition, have no similarity in non-*P. aeruginosa* species and other bacteria; therefore, they specifically detect only the *P. aeruginosa* strain. It should be noted that *Pseudomonas* species have similar quorum sensing system but the QS genes in each species are unique and conserved [37].

In this study, in all of the samples, the target sequences were amplified, which was parallel with the results of biochemical analyses and microbial cultures. Based on these analyses, the sensitivity of triplex PCR was high. Additionally in PCR reactions, using genomic DNA of non-*P. aeruginosa* species and other bacteria as templates, no false-positive results were observed, which demonstrates 100% specificity. In uniplex PCR, the primer sensitivity for the *las* gene was the same (10^{-4} DNA dilution), while the *gyrB* primers had the highest sensitivity (10^{-6} DNA dilution). In triplex PCR, the primer sensitivity was a 10^{-4} DNA dilution, which was similar to *lasI/R* uniplex PCR assays. For the first time, we are reporting the detection of this bacterium via the *las* and *gyrB* genes by a triplex PCR. In comparison to previous studies, these results showed that the selected genes are appropriate candidate targets for epidemiological purposes and regional

monitoring evaluation to achieve a common pattern of the prevalence of *P. aeruginosa*.

Finally, it should be noted that in recent years, quantitative PCR (qPCR) has been developed for the detection of this bacterium [10,21,38,39]. Although this type of PCR has advantages, such as speed, throughput, sensitivity and lower amounts of starting material, it also has some disadvantages, including the high cost of equipment, chemicals, and consumables and PCR inhibition [40]. In addition, due to its extremely high sensitivity, sound experimental design and normalization techniques are imperative for accurate conclusions. Additionally, in many laboratories, PCR in comparison to qPCR can be performed because it is a simple method with widespread availability.

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