ORIGINAL ARTICLE

PCR-based assay for the rapid and precise distinction of *Pseudomonas aeruginosa* from other Pseudomonas species recovered from burns patients

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Keywords

Pseudomonas aeruginosa • Burns patients • *OprL, OprI*

Summary

Background. *Pseudomonas aeruginosa is an important life-threatening nosocomial pathogen which plays a prominent role in wound infections in burns patients. We designed this study to identify the isolates of P. aeruginosa recovered from burns patients at the genus and species levels by means of primers targeting oprI and oprL genes.*
Methods. *During a 5-month period, wound samples were taken from burns patients and plated on MacConkey agar. All suspected colonies were screened for P. aeruginosa by means of a combination of phenotype tests. Specific primers for oprI and oprL genes were then used for the molecular identification of colonies.*
Results. *During the 5-month period, bacterial isolates recovered from burn wound infections were analyzed. Phenotype identification tests identified 171 (34.8%) P. aeruginosa isolates. However, molecular techniques that used species-specific primers to detect the amplicon of the oprL gene confirmed the exact identification of P. aeruginosa in only 133 cases; in the other isolates, the use of genus-specific primers detected the amplicon of the oprI gene, which confirmed the identification of fluorescent pseudomonads.*
Conclusions. *This study indicates that molecular detection by means of an assay targeting the oprL gene is a useful technique for the rapid and precise detection of P. aeruginosa in burns patients. In addition to phenotype testing, PCR detection should be carried out in order to promptly ascertain the best aggressive antibiotic therapy for P. aeruginosa infections, thereby significantly improving clinical outcomes.*

Introduction

Burn injury, one of the most common and devastating forms of trauma, is a major public health problem worldwide. Burn wounds can easily become infected because the skin no longer acts as an effective physical barrier against microbes. *P. aeruginosa* is the most common source of burn wound infections [1]. While these bacteria rarely cause disease in healthy individuals, they may do so in immuno compromised patients, such as those with AIDS or cystic fibrosis, and in burns patients [2]. The accurate identification of *P. aeruginosa* and detection of their susceptibility to antimicrobials are critical components of the management of burned patient. *P. aeruginosa* colonisation is normally detected by culturing wound swabs on artificial media. Typical isolation media used in wound infections include blood agar and chocolate agar and selective agars such as MacConkey agar and cetrimide-based media. Although large numbers of *P. aeruginosa* isolates are often present in clinical samples from burns patients, their detection and precise identification can often be challenging. For example, other species of Pseudomonas, as well as *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Ralstonia pickettii,* have been shown to grow on cetrimide-based media [3], and may be indistinguishable from non-pigmented strains of *P. aeruginosa*. Difficulties in recognizing *P. aeruginosa* are compounded by difficulties in biochemical identification. Biochemical test kits such as API 20 NE are commonly used for identification [4]; however, this technique has been seen to display a high rate of misidentification of oxidase-positive Gram-negative rods, including *P. aeruginosa* [5]. In addition, testing requires the use of a pure bacterial subculture and a minimum incubation time of 48 h. Hence, identification by means of this method requires at least 3 days. Another limitation of the conventional culture technique is that *P. aeruginosa* can easily be mistaken for closely related Gram-negative bacilli. The use of molecular techniques such as PCR could enable accurate and rapid identification of *P. aeruginosa* [6, 7]. L and I lipoproteins are two outer membrane proteins of *P. aeruginosa, and* are responsible for the inherent resistance of the bacterium to antibiotics and antiseptics. As these proteins are found only in this organism, they could be used as a reliable marker for the rapid identification of *P. aeruginosa* in clinical samples [8, 9]. In this study, we examined a technique for the rapid and precise identification of *P. aeruginosa* strains isolated from burns patients hospitalized in a main burns center in Iran. The performance of this technique, which utilizes PCR amplification of I lipoprotein (*OprI*) to detect the genus and

L lipoprotein (*OprL*) to detectthe species of *P. aeruginosa* strains, was compared with that of phenotypic and routine biochemical identification used in laboratories.

Materials and methods

Qualitative conventional detection

This study was carried out during a 5-month period, at a major center for the admission of burns patients in Tehran, Iran. Samples were obtained from burn wounds by swabbing. As in the routine phonotype tests usually performed in clinical laboratories, we inoculated burn wound swabs onto several selective media for the isolation of *P. aeruginosa*, including blood agar, MacConkey agar and Muller Hinton agar, and carried out incubation at 37ºC for 24-48 h. The isolates were presumptively identified by means of routine tests: colony morphology and pigment formation on selective medium, positive oxidase test, glucose fermentation, hydrolysis of gelatin and growth at 42ºC [10, 11].

Molecular (PCR) detection

DNA extraction. In order to minimize contamination and hence the possibility of false-positive results, all DNA isolation procedures were carried out in a room physically separated both from that used to set up nucleic acid amplification reaction mixtures and from the post-PCR room. Bacterial genomic DNA was extracted from all phenotypically and biochemically tested strains, as well as from the reference strain *P. aeruginosa* ATCC 27853, by means of a boiling method. For this purpose, depending on colony size, three to six colonies were picked from bacterial plates and mixed into 0.25 ml DNase/RNase-free water in sterile 1.5 ml eppendorf tubes in order to obtain a turbid suspension of bacteria (~ 1-2 × 109 cells/ml). The cell suspensions were kept in a boiling water bath for 10 minutes to lyse the cells, and were then centrifuged at 10000 g at 4°C for 10 minutes. Finally, the supernatant was transferred, in sterile conditions, into another tube and used as a DNA template. Extracted DNA was stored at -20°C prior to PCR amplification [12].

Primer selection. The primers used in this study are shown in Table I. PCR amplification of I lipoprotein (*OprI*) for the detection of *Pseudomonas* genus and L lipoprotein (*OprL*) for the detection of *P. aeruginosa* species was performed on all phenotypically tested strains of *P. aeruginosa*.

PCR amplification. In order to minimize contamination, all reaction mixtures were set up in a PCR room separate from that used for DNA extraction and amplification and from the post-PCR room. PCR was completed in adapted PCR micro centrifuge tubes according to the thermocycler used. Following optimization, reaction mixtures (25 µl) were set up as follows: 11 µl DNase/RNase-free water, 8 µl 2x PCR Master Mix (1.5 mM mgcl2, Denmark), 0.5 µl of each set of primers (*OprL* or *OprI*) and 5 µl of DNA template. The reaction mixtures were subjected to the following empirically optimized thermal cycling parameters in a thermocycler (Senso-Quest Labcycler, Germany): 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Positive (*P. aeruginosa* ATCC 27853 DNA) and multiple negative (water) amplification controls were included in every set of PCR reactions.

Detection of amplicons

Following amplification, aliquots (10 µl) were removed from each reaction mixture and examined by means of electrophoresis (80 V, 45 min) in gels composed of 1% agarose in TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.3). Gels were visualized under UV illumination by using a gel image analysis system (UVitec, Cambridge, United Kingdom) and all images were archived. Where a band was visualized at the correct expected size for *OprI*, the specimen was considered positive for *Pseudomonas* genus; if a band was visualized at the correct expected size for *OprL* loci, the specimen was considered positive for *P. aeruginosa* species.

Results

During the 5-month period, 491 bacterial samples recovered from burn wound infections were analyzed. The above-mentioned routine phenotype and biochemical tests enabled *P. aeruginosa* isolates to be recovered from 171 (34.8%) patients. By contrast, molecular techniques detected only 133 (27.08%) samples positive for *P. aeruginosa* species and 38 (7.73%) samples positive for pseudomonas genus. PCR assays employing each primer pair yielded DNA products of the predicted sizes (Fig. 1 and 2). The *OprI* and *OprL amplicon* genes were detected in all 133 *P. aeruginosa* isolates simultaneously. Table II shows the comparison of phenotype and biochemical testing with the molecular detection of *P. aeruginosa* in samples from burn wound infections.

Tab. I. Primers used in this study.

Primer	5′-sequence-3′	Product length (bp)	Reference
OprI-F	ATGAACAACGTTCTGAAATTCTCTGCT	249	7
OprI-R	CTTGCGGCTGGCTTTTTCCAG		
OprL-F	ATGGAAATGCTGAAATTCGGC	504	7
OprL-R	CTTCTTCAGCTCGACGCGACG		

Fig. 1. PCR amplification using Pseudomonas genus-specific primers (*Opr I* gene), M: marker, line 1: positive control, lines 2, 3, 4: clinical isolates of Pseudomonas genus, line 5: negative control.

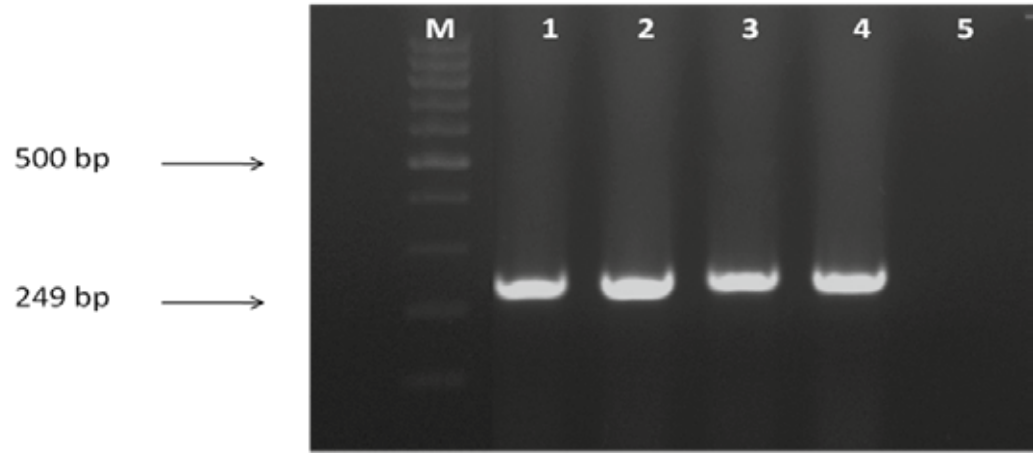
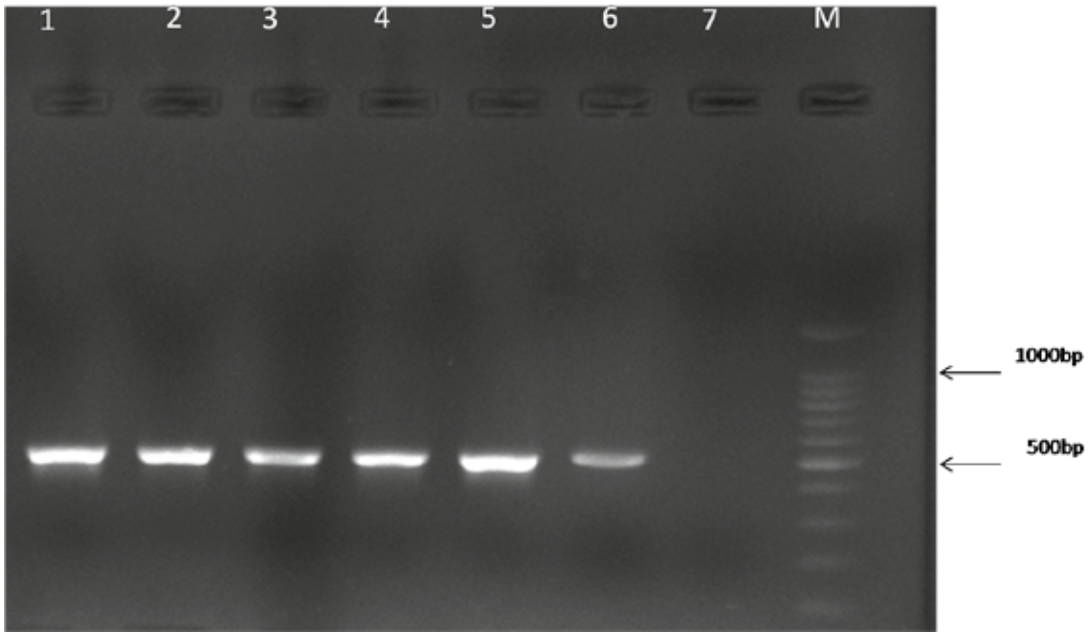


Fig. 2. PCR amplification using *P. aeruginosa*-specific primers (*Opr L* gene), M: marker, line 1: positive control, lines 2, 3, 4, 5, 6: clinical isolates of *P. aeruginosa*, line 7: negative control.



Tab. II. Comparison of phenotype and biochemical tests with molecular detection of *P. aeruginosa* in samples from burn wound infections.

Phenotypically & biochemically + (no. of isolates tested)	PCR(*OprI*)+ no. of isolates	PCR(*OprL*)+ no. of isolates
171	171	133

Biochemical method+: strains confirmed as *P. aeruginosa* on phenotype and biochemical testing.
PCR (OprI)+: strains confirmed as *Pseudomonas* genus by PCR amplification of *OprI*.
PCR (OprL)+: strains confirmed as *P. aeruginosa* genus by PCR amplification of *OprL*.

Discussion

Bacterial infections in burn wounds are common and are difficult to control. In recent decades, following the introduction of antibiotic therapy, *P. aeruginosa* has emerged as one of the most problematic Gram-negative bacteria in modern hospital settings. This organism is increasingly isolated as a nosocomial pathogen, and is responsible for high morbidity and mortality rates in burns patients, mechanically ventilated patients and those with cystic fibrosis [13, 14]. Infection by this bacterium is particularly problematic, since the organism is intrinsically

resistant to many drug classes and is able to acquire resistance to all effective antimicrobial drugs. Some studies carried out in Iran have also indicated that infections caused by multi-drug resistant (MDR) *P. aeruginosa* are widespread in Iranian hospitals [15, 16]. It is therefore important to identify *P. aeruginosa* accurately and rapidly and to ascertain the susceptibility pattern of this organism; this may avoid prolonged and sometimes unnecessary antibiotic treatments, which could select other antibiotic-resistant pathogens [12]. The identification of *P. aeruginosa* has traditionally relied on phenotypic and biochemical methods. These tests take a long time to perform and require extensive hands-on work by technicians, both for setup and for ongoing evaluation. Various methods have been developed to identify *P. aeruginosa* species rapidly and accurately. According to our literature review, PCR has the potential to identify microbial species rapidly and precisely through the amplification of gene sequences unique to a particular organism [17]. Indeed, several PCR-based, DNA probe methods have been developed to detect various pathogens in clinical samples and water and food samples [18]. Also in the case of *P. aeruginosa*, molecular methods have been reported to be superior to phenotypic methods in identifying *P. aeruginosa* species [19]. The outer membrane proteins of *P. aeruginosa* play important roles in the interaction of the bacterium with the environment [20]. In the present study, two PCR assays were performed individually for the molecular detection of two outer membrane lipoprotein genes, *oprI* and *oprL,* in samples from burn wounds [21]. According to the phenotype and biochemical tests carried out in this study, 171 (34.82%) of 491 bacterial samples recovered were classified as *P. aeruginosa*. However, while the results of our molecular tests *on oprI* and *oprL* genes confirmed that many of these isolatesbelonged to the *pseudomonas* genus, only 133 (77.7%) of them were confirmed as *P. aeruginosa* species. Thus, there was nearly complete agreement between molecular and conventional phenotype and biochemical detection techniques with regard to the attribution of the *Pseudomonas* genus, but not the *P. aeruginosa* species. This may account for the potential phenotype misidentification of *P. aeruginosa* which has been recently described [22]. Alternatively, discrepant results (PCR- /biochemical+) may emerge in the case of true *P. aeruginosa* colonization, in that a false negative culture result may be due to sample overgrowth by other bacteria, or to the presence of non-cultivable organisms or auxotrophic mutations of the organism. Indeed, it has been shown that *oprI* is conserved among members of fluorescent pseudomonads [23, 24]. De Vos et al. designed a multiplex PCR assay based on *oprI* and *oprL* genes for the molecular detection of *P. aeruginosa*, and showed that its specificity and sensitivity were 74 and 100%, respectively [7]. Lavenir et al. also noted that all 268 of the *P. aeruginosa* strains that they detected contained the *oprI* and *oprL* genes (sensitivity = 100%, specificity = 80%) [25]. This is in line with our findings. Although our PCR and DNA sequence analyses revealed some isolates that had been misidentified by phenotype testing, it must be said that our study was not designed to ascertain the frequency of misidentification of isolates from burn wound infections or to compare the relative accuracy of different phenotype identification systems. The isolates analyzed in this study constituted a biased set of atypical isolates that were difficult to identify. Nevertheless, these isolates were well suited to providing a rigorous test of our PCR assays and represented strains for which molecular analysis would be expected to be most useful. Our study also confirms that various non-aeruginosa pseudomonal species can occasionally be recovered from burn wound infection cultures. In this regard, genotype-based identification methods circumvent the problem of phenotype variation and provide more accurate species identification.

Conclusions

It is important that primary diagnostic bacteriology methods have the ability to detect transient and early Pseudomonas colonization in burns patients as soon as possible, so that: (i) aggressive antibiotic regimes may be reconsidered; (ii) the patient can be managed optimally, with a view to avoiding early biofilm formation and chronic colonization with *P. aeruginosa*, and (iii) appropriate infection control precautions can be taken.

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Authors' contributions

Maryam Adabi and Abbas Gholami conceived; designed and coordinated the research.
Mina Boostanshenas collected data. Mahshid Talebi-Taher and Ali Majidpour evaluated the results.
Maryam Adabi and Abbas Gholami wrote the manuscript. All Authors revised the manuscript and gave their contribution to improve the paper. All authors read and approved the final manuscript.

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