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Molecular Identification And Virulence Gene Patterns Of Pseudomonas Aeruginosa Isolates

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Abstract
The current research aimed to assess antibiotic resistance profiles and the detection of virulence factor genes, including lasB, and plcH among the clinical isolates of P. aeruginosa, and to identification of isolates by investigation of the rpoB gene. As well as determine whether a correlation exists between the prevalence of these virulence factors and antibiotic resistance in P. aeruginosa isolates. Pseudomonas aeruginosa infections are associated with significant morbidity and mortality due to the organism's ability to adapt easily to changes in the environment, rapidly develop resistance to antibiotics, and produce a variety of virulence factors. P. aeruginosa possesses a large number of virulence factors that may contribute to its pathogenicity, including the P. aeruginosa hemolytic phospholipase C (plcH), which degrades phosphatidylcholine, an abundant lipid in cell membranes and lung surfactant. A zinc metalloprotease called elastase lasB has an elastolytic activity on human tissue, and especially lung tissue. Phylogenetic characterization of the strains most often requires the use of certain household markers or phylogenetic markers, including subunit β RNA polymerase and 16S ribosomal RNA. A total of 50 isolates of P. aeruginosa were supplied from hospitalized burn patients. Identification and antimicrobial susceptibility tests were performed using the VITEK 2 system. Multiplex PCR was done to detect lasB, plcH, and rpoB genes. Multiplex PCR analyses of lasB, plcH, and rpoB genes showed that all P. aeruginosa isolates were positive. The most effective antimicrobial agents against clinical isolates were Meropenem, Amikacin, and Ciprofloxacin. The prevalence of lasB and plcH genes in clinical isolates plays an important role in the development of the disease. Also, phylogenetic markers rpoB used can be exploited for P. aeruginosa strains of different origin.
Keywords: *Pseudomonas aeruginosa, hemolyytic phospholipase C, elastase, RNA polymerase subunit β gene, multiplex PCR.*

1 INTRODUCTION

Pseudomonas aeruginosa is a non-fermentative, aerobic, Gram-negative rod-shaped bacterium (Fu et al., 2013). P. aeruginosa can secrete an elastase B (lasB) enzyme in response to environmental conditions (Lanotte et al., 2004; Hvorecny et al., 2018). Elastase B is an important protease of P. aeruginosa. This enzyme has a tissue-damaging activity, it can degrade a number of plasma proteins, and it contributes to the survival of P. aeruginosa in infected tissues (Lomholt et al., 2001; Nikbin et al., 2012). Phospholipases C (PLCs) are also implicated in virulence. P. aeruginosa produces three PLCs: one with hemolytic activity (PlcH), one other non-hemolytic (PlcN) and the last, a PLcB which is important for chemotaxis and plays an important role in "twiching" mobility (Mitov et al., 2010; Khattab et al., 2015; Fadhil et al., 2016; Ghanem et al., 2023). A phosphate deficiency induces the production of PlcH. These PLCs are secreted via the type-II secretion system (Khattab et al., 2015; Fadhil et al., 2016; Zakaria et al., 2019). Hemolytic phospholipase C (PlcH) is a secreted hydrolase that degrades host-associated phosphatidylcholine (PC) and sphingomyelin (Berka and Vasil, 1982; Vasil, 2006; Bomberger et al., 2009; Bogiel et al., 2023). These choline-containing phospholipids are abundant macromolecules in eukaryotic membranes and host lung surfactant. PlcH adversely affects the integrity of the lung and contributes to decreased lung function (Meyers et al., 1992; Wargo et al., 2009). Phospholipase C may also play a significant role in the phonation of skin lesions of P. aeruginosa (Lu, 1976). The purified hemolytic form plcH causes increased vascular permeability, end organ damage, and death when injected into mice in high doses (Berk et al., 1987; Meyers et al., 1992). The outer membrane proteins of P. aeruginosa OprI and OprL play important roles in the interaction of the bacterium with the environment as well as the inherent resistance of P. aeruginosa to antibiotics where the consequence of the presence of these specific outer membrane proteins that have been implicated in efflux transport systems that affect cell permeability (Nikaido, 1994). As these proteins are found only in this organism, they could be a reliable factor for rapid identification of P. aeruginosa in clinical samples (De Vos et al., 1997). Porins including OprM, OprN, OprJ, OpmG, OpmB, or OpmE are involved in the efflux of harmful molecules, including antibiotic drugs, thus

conferring antibiotic resistance (Auda et al., 2020; Irene et al., 2021). P. aeruginosa easily acquires additional resistance mechanisms, that leads to serious of therapeutic problems (Micek et al., 2005). Mucoid strains may yield biofilms, representing communities of attached microorganisms on a surface. Biofilms have a crucial part in infectious diseases. Supplementary, they have a favorable antibiotic resistance, with their matrix playing a major role (Jarjees, 2020). Phylogenetic characterization of the strains most often requires using of certain household markers or phylogenetic markers. These include rRNA 16S (ribosomal RNA), rec A (recombinase A), rpo D (σ70 factor RNA polymerase), gyr B (in β unit of DNA gyrase), rpo B (subunit β RNA polymerase) and ITS1 (spaceur intergenic transcribed) ("Internal transcribed Spacer") region between 16S-23S rDNA allow the differentiation of Pseudomonas species (Tampong et al., 2009, Benie et al., 2016; Chan et al., 2016). The present study was carried out in order to investigate the distribution of virulence genes and the pattern of antibiotic resistance of P. aeruginosa isolated from hospitalized patients who suffered from burn infections, as well as this research study was aimed to evaluate rpoB as reliable factors for rapid identification of P. aeruginosa isolates based on PCR amplification of RNA polymerase subunit β gene as phylogenetic marker for detection of this species.

2 MATERIALS AND METHODS

2.1Samples Collection

A total of 50 isolates of P. aeruginosa were recovered from wound burn samples.

2.2Isolation and Identification of P. aeruginosa

All isolates were identified as P. aeruginosa based on colonial morphology, Gram staining, growth at 42 °C, the presence of characteristic pigments, biochemical tests including oxidase test and catalase test, as well as microbial identification was accomplished on the VITEK 2 automated system. P. aeruginosa ATCC 27853 was used as a standard strain in this study. All isolates were incubated in Tryptic Soy Broth containing 30% glycerol at -80 °C.

2.3Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was performed on the VITEK 2 automated system (bio Merieux) for the following antimicrobials: Ticarcillin, piperacillin, Ticarcillin/Clavulanic Acid, Piperacillin/Tazobactam, Ceftazidime, Meropenem, Cefepime, Amikacin, Imipenem, Gentamicin, Tobramycin, Ciprofloxacin.

2.4Bacterial Genomic DNA Extraction

The bacterial cells were cultured in Tryptic Soy Broth (Merck, Germany) and further incubated for 48 h at 37°C. The genomic DNA was extracted from p. aeruginosa colonies using the DNA extraction kit (Geneaid/Korea) according to the manufacturer's instructions. The extracted DNA was subjected to PCR reactions targeting LasB, plcH, and rpoB genes.

2.5DNA Purification

The extracted DNA was checked by measuring the optical density (OD) at 260 nm and 280 nm using a spectrophotometer. The DNA extracted samples were stored at -20 °C until being used.

2.6Molecular Analysis for LasB, plcH and rpoB genes with polymerase chain reaction (PCR)

The primer sequences and amplification protocols followed the indications provided by (Benie et al., 2017), Table 1. The uniplex Polymerase chain Reaction used for amplification genes specific targeted sequence in a thermocycler machine (Techne/UK) for the identification of lasB, plcH, and rpoB. The master mix preparation was done in a total volume of 25µl (12.5 µl Gotaq Green Master Mix (Promega/USA), 3µl of genomic DNA, 1 µl of each lasB and plcH primer separately, and 7.5µl µl of free water). The same program cycle used for lasB and plcH genes, the amplification reaction started by heating at 95°C for 5 min before thermocycling, DNA denature at 94°C for 35 sec, primer anneals at 60°C for 1 min, extensions at 72°C for 1 min and the mixtures were held at 72°C for 7 min after the finishing 35 cycles.

On the other hand, all the strains were subjected to amplification rpoB gene. The PCR premix for rpoB consists of a total volume of 25µl, 12.5 µl Gotaq Green Master Mix (Promega/USA), 3µl of genomic DNA, 1.5 µl of each primer, and the volume is completed with 6.5µl µl of free water. The PCR was done according to the following conditions: initial denaturation at 94°C for 3min. The 35 cycles amplification for denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension step at 72°C for 1 min, then the final extension at 72°C for 7 min. Eventually, the PCR product amplicons were analysed by agarose gel electrophoresis (2%) and the DNA stained with Safe dye (Bioland/USA). The presence and

absence of the virulence and phylogenetic marker genes were determined using UV transillumination (Syngene/UK), and the PCR product band for each of the lasB, plcH, and rpoB genes was identified on the gel as compared to the standard DNA ladder/1kb (Norgenbiotek/Canada).

Table 1: The oligonucleotide sequences for the phylogenetic marker and virulence genes.

Target gene	Primer sequences (5 ' - 3 ')	PCR product size	Reference
LasB F **LasB R**	 5'GGA ATG AAC GAG GCG TTC TC3' 5'GGT CCA GTA GTA GCG GTT GG3'	300bp	
plcH F **plcH R**	5' GAA GCC ATG GGC TAC TTC AA 3' 5' AGA GTG ACG AGG AGC GGTAG 3'	307 bp	(Benie et al., 2017)
rpoB F **rpoB R**	5'CAG TTC ATG GAC CAG AAC AAC CCG 3' 5'ACG CTG GTT GAT GCA GGT GTT C 3'	759bp	

3 RESULTS AND DISCUSSION

3.1Results

According to the morphology of the colony, the isolated colony was identified, including the presence of characteristic pigments and growth at 42°C. P. aeruginosa does not ferment lactose, can easily be differentiated from lactose-fermenting bacteria on MacConkey agar, as well as isolates were identified biochemically, including oxidase and catalase positivity. All P. aeruginosa isolates were identified and screened for 12 antimicrobial agents by the VITEK 2 compact system. In the present study, all isolates were found to be resistant to Ticarcillin, Ticarcillin/Clavulanic Acid, Piperacillin/Tazobactam, Piperacillin, Ceftazidime, and Cefepime. On the contrary, resistance to Imipenem, Meropenem, Amikacin, Gentamicin, Tobramycin, and Ciprofloxacin was 5%, 5%, 5%, 7%, 7%, and 5% respectively. In addition, intermediate resistance to Imipenem and Gentamicin was 9% and 7%. The results showed that all bacterial isolates carried the rpoB gene. This technique was performed for rapid and confirmed identification of this species, also the results found that all bacterial isolates harbored lasB and plcH virulence genes Figures 1 and 2.

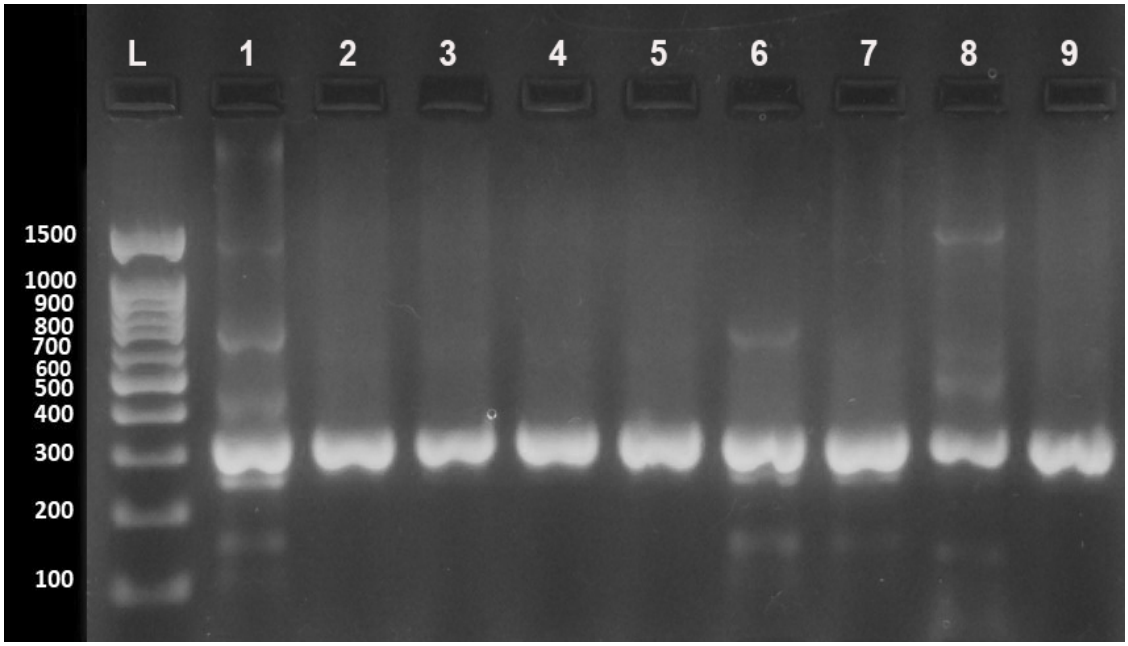

Figure 1. Electrophoresis of the PCR products of lasB gene. Lane 1: Ladder of 100bp, Lane 1 -9: lasB gene identification (300bp).

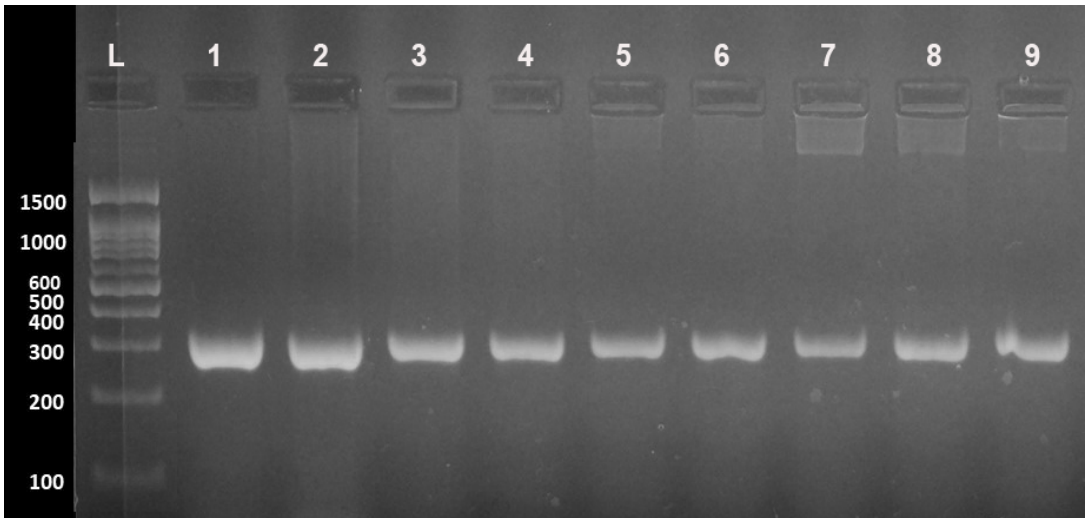

Figure 2. Electrophoresis of the PCR products of plcH gene. Lane 1: Ladder of 100bp, Lane 1 -9: plcH gene identification (307bp).

3.2DISCUSSION

Molecular identification by using the rpoB gene showed that all of the strains were P. aeruginosa. This result also indicated the strong discriminating power of the identification method using the rpoB gene and confirmed the heterogeneity of the P. aeruginosa observed by some authors. (Ait-Tayeb et al., 2005; Benie et al., 2016; Benie et al., 2017). Molecular methods have been reported to be superior to the phenotypic methods for identification of P. aeruginosa (Khattab et al., 2015; Gholami et al., 2016). This high molecular identification rate showed that genomic studies are needed to confirm the exact taxonomic position of P. aeruginosa. Further, the quality and purity of nucleic acids are among the most critical factors for PCR analysis. (Urakawa et al., 2010). In this research study, all tested isolates harbored the lasB gene, which is in agreement with previous studies (Mitove et al., 2010; Nikbin et al., 2012; Khatab et al., 2015; Aljaafreha et al., 2019). The lasB is one of the most important proteases of P. aeruginosa (Lomholtet et al., 2001). Mutation of the lasB gene markedly reduces P. aeruginosa invasion. Prevalence of the lasB gene in all the environmental and clinical isolates implies the importance of the LasB factor to the survival of P. aeruginosa in various settings (Cowell et al., 2003). The present study found that all P. aeruginosa carried the plcH gene, and similar results have been recorded by Lanotte et al, in French, reported that PCR detected algD, lasB, toxA, plcH, plsN, and nan2 in all of the 162 isolates studied, which were obtained from sputa of CF patients, clinical samples from patients without inherited disease, and plants. The plcH gene is responsible for proinflammatory activities, virulence in animal models, pulmonary inflammation, and inhibition of oxidative burst of neutrophils (Terada et al., 1999; Wieland et al., 2002; Idris et al., 2012). Ciragil et al. studied the elastase, protease, and alginate properties of P. aeruginosa strains isolated from different parts of the body. The differences in the distributions of virulence factor genes in the populations strengthen the probability that some P. aeruginosa strains are better adapted to the specific conditions found in specific infectious sites, and thus, virulence gene expression differs according to site and severity of infection (Nikbin et al., 2012). Determination of different virulence genes of P. aeruginosa isolates suggests that they are associated with different levels of intrinsic virulence and pathogenicity. This may have different consequences on the outcome of infections. Significant correlations between some virulence genes and source of infection obtained in this research indicate implementation of infection control measures will help in controlling the dissemination of virulence genes among P. aeruginosa isolates. The expression of exotoxin A, proteases, and hemolysins is controlled by quorum sensing systems (Pearson et al., 1994; Pearson et al., 1997; Whiteley et al., 1999). Beveridge's group has characterized bacterial-derived outer membrane vesicles (OMV) to be a novel secretion mechanism employed by bacteria to deliver various bacterial proteins and lipids into host cells, eliminating the need for bacterial contact with the host cell (Nguyen et al., 2003). Beveridge's group and others have reported that some secreted virulence factors from P. aeruginosa, including β-lactamase, hemolytic phospholipase C, alkaline phosphatase, pro-elastase, hemolysin, and quorum sensing molecules, like N-(3-oxo-dodecanoyl) homoserine lactone and 2-heptyl-3-hydroxy-4-quinolone (PQS), are

also associated with P. aeruginosa OMV(Kuehn and Kesty, 2005; Montes et al., 2007; Bomberger et al., 2009; Abbas et al., 2018). The expression of the las system relies on environmental stimuli such as iron (Bollinger et al, 2001), osmolarity (Chopp et al., 2003), nitrogen and oxygen availability (Wagner et al., 2003). 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 (Girard and Bloemberg, 2008; Rutherford and Bassler, 2012: Qin et al., 2022; Muggeo et al., 2023). These genes are expressed only when a high cell density is achieved (Pesci et al., 1997; Seed et al., 1995). This controlled transcription of genes, in the course of a rapid adaptation to environmental challenge, is essential for bacterial survival and for the promotion of chronic infection. All these factors have been described to contribute to the virulence of P. aeruginosa in vitro (McMorran et al., 2003), in animal models (Johansen, 1996), and in clinical studies (Berthelot et al., 2003). Hence, judicious use of antibiotics is required by clinicians. It is compulsory to evaluate the prevalence of virulence factors and the pattern of antibiotic resistance among clinical isolates of P. aeruginosa strains. P. aeruginosa can develop resistance to antibiotics because of the low permeability of its outer membrane, the constitutive expression of various efflux pumps (Livermore, 2001), and the naturally occurring chromosomal AmpC β-lactamase, turning it resistant toward penicillin G, aminopenicillins, and first- and second-generation cephalosporin (Nordmann and Guibert, 1998; Hasanpour et al., 2023). The rpoB gene was used to characterize spontaneous mutations in the chromosome of P. aeruginosa and P. putida (Jatsenko et al., 2015). The highly conserved rpoB gene, which encodes the B subunit of RNA polymerase, is the target of mutations leading to rifampicin resistance (Rifr) in both Pseudomonas spp. The rpoB/Rif^r system senses base substitutions that cause amino acid changes in the central rifampicin binding pocket (cluster I–III) or the N-terminal cluster in RNA polymerase (Juurik et al., 2012; Mariela et al., 2013). Treatment of bacterial infections is complicated by the ability of bacteria to develop resistance to antibiotics. Acquired resistance arises either by mutation or via horizontal transfer of resistance genes from other organisms.

CONCLUSIONS

In conclusion, the use of rpo β genes provides more confident detection of P. aeruginosa by PCR. Findings of the present study show the importance of virulence genes related to type II secretion systems, including lasB, and plcH, on clinical isolates and Further studies with a large sample size and expression rate analysis are required to determine the actual role of these virulence genes in different clinical infections caused by P. aeruginosa. However, a regular surveillance of virulence determinants, monitoring of antibiotic susceptibility patterns, and designing a practical guide for antibiotic stewardship for P. aeruginosa are suggested.

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