Study on pyoverdine and biofilm production with detection of LasR gene in MDR Pseudomonas aeruginosa

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Abstract

In cystic fibrosis individuals, chronic lung infections and hospital-acquired pneumonia are caused by Pseudomonas aeruginosa. P. aeruginosa generates siderophores such as pyoverdine (PVD) as iron uptake systems to cover its needs of iron ions for growth and infection. lasR quorum sensing (QS) gene has a crucial function in PVD production and biofilm generation in P. aeruginosa. Fifty isolates of P. aeruginosa were obtained from clinical specimens of sputum (collected from individuals suffering from pulmonary infections). Antibiotic sensitivity test was performed for 50 P. aeruginosa isolates by using 10 different types of antibiotics. All isolates of P. aeruginosa showed resistance for all 10 using antibiotics in this study. Ten multidrug resistant isolates of P. aeruginosa were selected for next tests. Virulence factors of ten multidrug resistant isolates of P. aeruginosa, such as biofilm generation, PVD production, and lasR gene were detected. From results, all 10 P. aeruginosa isolates can produce biofilm, PVD, and contain lasR gene. The produced amplicon for the lasR gene was 725 bp. After mice injection by fresh and heated PVD produced by P. aeruginosa PS10 LCG19328.2, the fresh PVD caused 100% mortality within five days using 0.3 ml of its concentration (37.4 μM), while (15.3 μM) of heated PVD (toxoid) caused 50% mortality.

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacteria that can induce severe morbidity and death in hospitalized patients. It leads to massive nosocomial and healthcare-correlated disorders. Its antibiotic resistance, virulence characteristics, and capacity to cope with a wide range of conditions increase the problem. P. aeruginosa has evolved into a public health issue that requires immediate attention and management (Spagnolo et al., 2021).

In individuals with cystic fibrosis and other lung diseases, P. aeruginosa is the most prevalent pathogen inducing long-term infections. When bacteria stick to a surface that is conducive to the formation of a biofilm matrix, they can establish a robust biofilm structure. Because of their various tolerance mechanisms, these bacterial biofilms have a greater natural resistance to standard antibiotic therapy. As a result of the current situation, the number of medication impairments linked with the infections of P. aeruginosa biofilm is on the rise (Vetrivel et al., 2021).

P. aeruginosa produces PVD as a siderophore that is required for the pathogenesis of human diseases, as well as the acquisition of iron and/or the regulation of other virulence factors. PVD-mediated iron separation impairs host mitochondria by altering their function and inducing autophagy-mediated turnover of mitochondria. The ESRE network, which is regulated by the host, identifies this destruction through a conserved mitochondrial surveillance mechanism. The research clarifies PVD’s pathogenic mechanisms and emphasizes its role in host-pathogen interactions (Kang et al., 2018).

To produce infections, virulence components are essential. The spatial organization of a population, including a biofilm, has been shown to boost the generation of several virulence factors, notably pyoverdine, which is generated by P. aeruginosa (Quinn et al., 2021).

Throughout infections, quorum sensing (QS) is critical for P. aeruginosa pathogenicity. It is involved in the generation and...
growth of biofilms and controls the generation of virulence factors. QS has been correlated with antibiotic resistance and has been hypothesized to have a function in \textit{P. aeruginosa}'s capacity to establish infection and remain in a host. The generation of various survival and virulence functions in \textit{P. aeruginosa} is regulated by three separate but interconnected QS systems (\textit{rhl, las, and pqs}). The full stimulation of the \textit{rhl} and \textit{pqs} systems is dependent on the \textit{las} system and its transcriptional regulator LasR. In \textit{P. aeruginosa}, a deficiency in \textit{las} activity seems to be a common adaptation method (Groleau et al., 2021).

Blocking the formation of pyoverdine using fluoroypyrimidines is one way to compromise pyoverdine (Kirienko et al., 2016) or its role utilizing gallium (Ga3+) or pyoverdine suppressor (Kirienko et al., 2019), is effective in preventing \textit{P. aeruginosa} pathogenesis, indicating that pyoverdine could be used as targeted therapy. Individuals from three separate groups of pyoverdine antagonists increased lifespan considerably (fluoroypyrimidines, gallium, and LK11). As the effectiveness of anti-microbial declines, addressing virulence factors generally, and pyoverdine, in particular, has been highlighted as a potential technique for reducing bacterial pathogenicity (Kang et al., 2019).

Toxoid vaccines are created by purifying bacterial exotoxin and then using heat to inhibit or inactivate it (while maintaining immunogenicity), anti-toxoid antibodies are produced in response to toxoids, and they attach to the toxin and negate its consequences. To accomplish detoxification or inactivation without substantial change of the antigenic epitope structure, toxoid vaccine production techniques must be closely managed. This approach is restricted for disorders in which produced poisons are the primary illness induction. After receiving a vaccine (detoxified toxins) comprising a harmless toxoid, the immune system can learn to combat the natural toxin by creating antibodies that opsonize bacterial toxins (Yadav et al., 2020).

This study aimed to survey MDR samples of \textit{P. aeruginosa} derived from pulmonary infections and indicated an effective, positive relation between biofilm and production of virulence factors such as pyoverdine (PVD) and aimed to study the presence of \textit{lasR} Q5 gene with its control on virulence factors. Moreover, it was focused on toxin neutralization for the treatment of PVD to toxoid form as a recent advances anti-virulence therapeutics to stimulate or activate the immune system and to decrease the mortality of mice.

2. Materials and methods

2.1. Bacterial strains

Fifty isolates of \textit{Pseudomonas aeruginosa} were obtained from clinical specimens of sputum (collected from individuals suffering from pulmonary infections at Zagazig University Hospital, El-Sharkia, Egypt) on cetrimide agar (Brown and Lowbury, 1965).

2.2. Antibiotic sensitivity isolated \textit{Pseudomonas aeruginosa}

Antibiotic susceptibility test was conducted on \textit{P. aeruginosa} isolates using ten different antibiotics by disc diffusion technique (Cursino et al., 2005) as per clinical and laboratory standards institute's authorized technique (Table S1). In all antibiotic disc susceptibility tests, Mueller-Hinton agar was utilized as a growing medium.

2.3. Quantitative assay for biofilm by tissue culture plate approach (TCP)

Biofilm assessment was determined for ten MDR \textit{Pseudomonas aeruginosa} isolates. A quantitative test described by Bose et al., (2009) was performed as a gold-standard strategy for biofilm determination. According to this method, bacterial biofilms were grown in LB medium on 96-well microtiter plates at 35 °C. Incubated microtiter plates were incubated at 35 °C for eight hours before rinsing to eliminate planktonic bacteria. Wells which were filled with growth medium served as negative controls. The plates were stained with 0.1 percent crystal violet for five minutes. By rinsing the plates three times with distilled water, the crystal violet solution was eliminated. The stained adhering biofilm OD was measured using a micro-ELISA auto reader at 570 nm. When the cut-off belonged to non-adherent (OD less than 0.120), the isolates were categorized as negative biofilm producers, when the cut-off was related to weakly or strongly adherent (OD = 0.120 – 0.240 or > 0.240), the samples were classed as positive biofilm producers.

2.4. Pyoverdine production

Pyoverdine was derived from \textit{P. aeruginosa} isolates utilizing the approach of Saha et al., (2008) with modification. For pigment synthesis, the chosen MDR \textit{P. aeruginosa} samples were cultured in Brain Heart Infusion broth at 37 °C for four days. The supernatant was collected after the pigment-rich broth culture (5 ml) was centrifuged twice at 5000 rpm for 30 min. It was then filtered using filter paper with a pore size of 0.45 μm and used as a crude extract. The used solvent used to dissolve the filtrate was 1:1 combination of chloroform and methanol (v/v). The pyoverdine was obtained by separating the funnel three times, and then collected in vials. PVD was extracted with a tiny amount of 50 % (vol/vol) methanol, evaporated, and dissolved in distilled water. PVD level in supernatants was measured as the OD 405 (Imperi et al., 2009). The PVD concentration was determined by spectrophotometric employing the molar extinction co-efficient 1.4 × 104 M − 1 cm − 1 at OD 405 nm (James et al., 2005).

2.5. Molecular studies

2.5.1. DNA extraction

Cultured colonies were subjected to DNA extraction using the ZymoResearch Quick-gDNA™ MiniPrep kit, Catalog No. D3024.

2.5.2. \textit{lasR} quorum sensing gene detection in \textit{P. Aeruginosa} isolates by PCR

The forward primer (5’-ATGGCTTGTTGACGGT-3’) and reverse primer (5’-GCAAGACGAGATAAAGACCA-3’) were used to amplify the \textit{lasR} gene according to Schaber et al., (2004). Gel control DNA marker was used in test with different size of bands 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp.

2.5.3. Identification of selected \textit{P. Aeruginosa} isolate

The identification of the most efficient producer \textit{Pseudomonas aeruginosa} PS10 for biofilm and PVD was confirmed using 16S rDNA sequencing (Cibik et al., 2000). The isolate was subjected to sequence analysis by the basic local alignment search tool program (Figure S1), and submission was made in GenBank. The 16S forward primer (5’-AGAGTTTGTATCCTCGTAG-3’), and the 16S reverse primer (5’-GGTACCTGTGTTAGACTT-3’) were used for DNA amplification.

2.6. Immunological investigations

2.6.1. Preparation of fresh and heated PVD produced by \textit{P. Aeruginosa} PS10

Purified PVD of \textit{P. aeruginosa} PS10 (grown in medium with 25 mM conc. of iron at 37°C for three days) was heated at 115°C for 20 min to prepare the toxoid (Eraso and Albesa, 1996). The con-
centration of the fresh (toxin) and heated (toxoid) PVD were recognized as previously mentioned by James et al., (2005).

2.6.2. Mice toxicity of the fresh and heated PVD by P. Aeruginosa PS10

Each group of four male Swiss albino mice (6–8 weeks old) were injected intraperitoneal (i.p) with the purified PVD toxin (A, B and C groups) and toxoid (D, E and F groups). The injection doses were 0.5, 0.3, and 0.1 ml of the purified PVD toxin or toxoid (M). The toxicity, as reflected by the mortality of mice, was scored at five days after the injection, and the lethal dose of the tested, and purified PVD was determined. According to Abraham (1992), the mortality level was evaluated utilizing the formula as follows:

\[
\text{Mortality level} = \frac{\text{Died animals number}}{\text{Overall number of animals per group}}
\]

2.7. Statistical analysis

The obtained data were statistically analyzed employing the one-way ANOVA test. All statistical analysis was conducted by IBM SPSS Corp. The significance of the discovered results was calculated (p-value less than 0.05). Each value of the collected findings is the standard deviation of the mean of three replicates.

3. Results

3.1. Antibiotic susceptibility of P. Aeruginosa isolates

The resistant percentage of the antibiotics (imipenem, levofloxacin, colistin, meropenem, amikacin, piperacillin, ceftazidime, gentamicin, ciprofloxacin, and aztreonam) against tested P. aeruginosa was tabulated in (Table 1)( Table S2).

The result indicated that all isolates were resistant to imipenem, piperacillin, and ceftazidime, while high susceptibility to meropenem and colistin was shown. Also, it was observed that ten isolates represented high resistance percentages to all used antibiotics, and they were selected as multidrug-resistant isolates for further investigation.

| Antibiotics  | Pseudomonas aeruginosa isolates |
|--------------|---------------------------------|
|              | Sensitive | Resistant | Sensitive | Resistant |
| Imipenem (IPM) | 0 | 0 | 50 | 100 |
| Levofloxacin (LEV) | 30 | 60 | 20 | 40 |
| Colistin (CT) | 35 | 70 | 15 | 30 |
| Meropenem (MEM) | 46 | 88 | 4 | 8 |
| Amikacin (AK) | 22 | 32 | 28 | 56 |
| Piperacillin (PP) | 0 | 0 | 50 | 100 |
| Ceftazidime (CAZ) | 0 | 0 | 50 | 100 |
| Gentamicin (CN) | 14 | 18 | 36 | 72 |
| Ciprofloxacin (CIP) | 31 | 42 | 19 | 38 |
| Aztreonam (ATM) | 36 | 48 | 14 | 28 |

Table 1 Variations of anti-microbial susceptibility in Pseudomonas aeruginosa isolates.

| Antibiotics  | Sensitive | Resistant |
|--------------|-----------|-----------|
| Imipenem (IPM) | 0 | 0 |
| Levofloxacin (LEV) | 30 | 60 |
| Colistin (CT) | 35 | 70 |
| Meropenem (MEM) | 46 | 88 |
| Amikacin (AK) | 22 | 32 |
| Piperacillin (PP) | 0 | 0 |
| Ceftazidime (CAZ) | 0 | 0 |
| Gentamicin (CN) | 14 | 18 |
| Ciprofloxacin (CIP) | 31 | 42 |
| Aztreonam (ATM) | 36 | 48 |

Chi test p-value

- significant deference at level 0.01
- ns: not significant deference.

3.2. Biofilm assay and PVD production

The result showed that all isolates were biofilm producers, 20 % moderately biofilm producers, and 80 % strongly biofilm producers. The result showed that 100 % of isolates were a producer for PVD siderophore (Table 2).

The result showed that isolate PS7 was the weak producer for PVD and biofilm, while isolate PS10 was the strong producer. Moreover, it showed that the more biofilm formation, the more PVD production, and vice versa (Figure S2).

3.3. Detection of lasR QS gene in P. Aeruginosa isolates

It was shown that all tested P. aeruginosa isolates were positive for the lasR quorum-sensing gene by PCR, as shown in (Fig. 1). The result indicated that the produced amplicon for the lasR gene was 725 bp.

The prior outcomes found that all 10 MDR P. aeruginosa isolates were producers for biofilm, PVD, and harbored the lasR QS gene. The biofilm and PVD producer PS10 was selected for further investigations.

3.4. Molecular identification of selected P. aeruginosa isolates

Identification of P. aeruginosa PS10 was confirmed as Pseudomonas aeruginosa (Table S3), by PCR amplification of 16S rRNA gene. The partial nucleotide sequence of the amplified gene from Pseudomonas aeruginosa was submitted in GenBank with accession number LC619328.2. The phylogenetic tree was indicated in (Fig. 2).

3.5. Immunological investigations

3.5.1. Fresh and heated purified PVD by P. Aeruginosa PS10

The concentration of both fresh (toxin) (Figure S3) and heat-treated (toxoid) PVD by P. aeruginosa PS10 were determined by spectrophotometric employing the molar extinction co-efficient (e) 1.4 \times 10^{-4} M^{-1} cm^{-1} at OD 405 nm.

As shown in (Table 3), the result detected that the concentration of fresh PVD was (37.4 \mu M), while the concentration of heated PVD was (15.3 \mu M) after heating at 115°C for 20 min. Thus, the heated...
Table 2
Quantitative determination of the biofilm production and PVD production by 10MDR P. aeruginosa isolates.

| Pseudomonas aeruginosa | Biofilm production | PVD production |
|------------------------|--------------------|----------------|
|                        | OD570 nm           | OD 405 nm      | Conc. (µM) |
| PS 1                   | 0.504 ± 0.004b     | 0.30 ± 0.003b  | 21.4       |
| PS 2                   | 0.394 ± 0.002d     | 0.26 ± 0.003d  | 18.5       |
| PS 3                   | 0.354 ± 0.004e     | 0.23 ± 0.005e  | 16.4       |
| PS 4                   | 0.304 ± 0.004f     | 0.18 ± 0.004g  | 12.8       |
| PS 5                   | 0.334 ± 0.004f     | 0.20 ± 0.004f  | 14.3       |
| PS 6                   | 0.461 ± 0.004c     | 0.28 ± 0.004c  | 20         |
| PS 7                   | 0.132 ± 0.004k     | 0.12 ± 0.004k  | 8.57       |
| PS 8                   | 0.284 ± 0.004h     | 0.14 ± 0.003h  | 10         |
| PS 9                   | 0.224 ± 0.004j     | 0.13 ± 0.004j  | 9.28       |
| PS10                   | 0.524 ± 0.004a     | 0.33 ± 0.004a  | 23.5       |

No biofilm (less than 0.120), Moderate (0.120–0.240), strong (>0.240).

One-way ANOVA and LSD were used to analyze the data. The data are the standard error of the mean of three replicates. P-value is less than or equal to 0.05–0.01 for significant differences between means in the same column. There is no significant difference between means in the same column.

PVD showed decreasing of concentration after heating treatment (Figure S4).

3.5.2. Mice toxicity of the fresh and heated PVD produced by P. Aeruginosa PS10

As reflected by the mortality in mice injected intraperitoneally by PVD before and after heating, the toxicities are shown in Table 4.

It was found that heat treatment disrupted spectral properties of PVD (fresh) after heating at 115°C for 15 min. The fresh PVD caused 100 % mortality within five days using 0.3 ml of its concentration (37.4 µM). In contrast, 15.3 µM of heated PVD (toxoid) caused 50 % mortality.

4. Discussion

Pseudomonas aeruginosa has been linked to nosocomial infections, and it is accounted for the majority of hospital-acquired diseases around the world. It remains one of the therapeutic concerns owing to the increased morbidity and mortality rates correlated with it, as well as the risk of developing drug resistance through output treatment. Due to the rise in drug resistance, traditional antibiotic regimens against P. aeruginosa have become increasingly ineffective (Chatterjee et al., 2016).

In this study, it was screened 50 Pseudomonas aeruginosa isolates from sputum in pulmonary infection patients that showed multi drug resistance for all used 10 antibiotics type.

Only one retroactively study documented that PA infection occurred regardless of lung function impairment and that MDR strains infected 11 % of patients (McDonnell et al., 2015).

One hundred and forty-seven (19.7 %) from 747 bronchiectasis patients had P. aeruginosa isolate in the sputum or bronchoscopic culture. PA-R accounted for 88 (59.9 %) while PA-sensitive was 59 (31.1 %) patients (Gao et al., 2018).

For at least one isolate from 934/983 (95 %) patients (228 children) culturing P. aeruginosa from their sputum were showed antibiotic resistance data (Smith et al., 2015).

A total of 80 clinical P. aeruginosa isolates showed resistance to all antibiotics. Rate Against amikacin and piperacillin/tazobactam was 12.5 % (n = 10), while was 23.75 % (n = 19) to levofloxacin. 20 % (n = 16) from isolates were multidrug-resistant P. aeruginosa in addition to 83.75 % (n = 67) of isolates showed biofilm formation. In 87.5 % (n = 70) of P. aeruginosa was found all three biofilm-related genes simultaneously while 13.5 % (n = 10) of the isolates had none of the genes tested. It can be overcome Pseudomonas infections by combination therapy such as an anti-pseudomonal beta-lactam (piperacillin/tazobactam or ceftazidime) and an aminoglycoside or carbapenems (imipenem, meropenem) with fluoroquinolones in conjunction with an aminoglycoside. However, reasonable antimicrobial use and high standards of infection prevention and control are essential to prevent further development of antimicrobial resistance. There are strategies based on the compatible antipseudomonal antibiotics along with anti-biofilm agents that can also be selected to destroy biofilm-associated infections (Kamali et al., 2020).

P. aeruginosa is classified as an opportune nosocomial bacterial pathogen that causes persistent infections due to its propensity to build a biofilm. In P. aeruginosa, biofilm is a significant virulence component that assumes a significant role in antibiotic resistance and persistent burn wound infections (Banar et al., 2016; Rodríguez-Lucas et al., 2020).

In this study, biofilms assay using TCP method for 10 MDR P. aeruginosa were measured, and the result showed that 0 % (0/10) non-producer biofilm, 20 % (2/10) moderately biofilm producers, and 80 % (8/10) strongly biofilm producers.
In this relation, the results of biofilm formation by the Congo red agar and the tissue culture plate strategy showed that 12% (6/50) produced biofilm, and 88% (44/50) of $P. aeruginosa$ isolates were not biofilm producers (Mahmoud et al., 2021).

In another study, 77.5% (31/40) of $P. aeruginosa$ isolates were classified as biofilm producers using a quantitative approach for evaluating biofilm development. A phenotypic assay was regarded as a "gold standard" for biofilm recognition as follows: weakly adherent 42.5% (17/40), moderately adherent 27.5% (11/40), and firmly adherent 7.5% (3/40) (Lima et al., 2018).

Bacteria manufactured siderophores (iron-chelating compounds) to collect iron from the host when the iron is scarce (Gomila et al., 2018). $P. aeruginosa$ produces two forms of siderophores; pyoverdine, which has a great affinity for Fe$^{3+}$ ions, and pyochelin, which has a low affinity (Abbas et al., 2018).

In this study, 10 MDR $P. aeruginosa$ were tested for production of pyoverdine pigment, and the result showed that 100% (10/10) was the producer for PVD siderophore.

**In a study by Kang et al. (2019), they looked at developing PVD, a major virulence factor, in 70 Pseudomonas aeruginosa isolates from children with cystic fibrosis, and found a link between pathogenicity in Caenorhabditis elegans and an acute mouse pneumonia model. PVD accumulation was seen in host tissues, particularly C. elegans extrapharyngeal tissues and mice lung tissues, in which accumulation was linked to host death.**

Several researchers reported siderophore secretion by various microorganisms (Sayyed et al., 2005; Omidvari et al., 2010; Sasirekha and Srividya, 2016). In succinate medium, $P. aeruginosa$ FP6 generated 85.7 μM siderophore (Sasirekha and Srividya, 2016). In $P. aeruginosa$ FP6, spectrophotometric examination of the culture in a conventional succinate medium revealed a peak between 420 and 450 nm that indicated the existence of a ferric hydroxamate siderophore (Ali and Vidhale, 2011). The quantity of hydroxamate siderophore secreted was 18.18 mg/L, while (Gull and Hafeez, 2012) noticed 15.5 μg/mL of hydroxamate siderophore in $P. fluorescens$ Mst 8.2. (Chandra et al., 2007), recorded the hydroxamate from siderophore was 32 μg/mL by Mesorhizobium loti after 48 hr of incubation.

Six genes encoding proteins required for pyoverdine production have previously been discovered in the Pseudomonas aeruginosa strain PAO1. An alternate sigma factor PVDs is needed to express all of these genes. In the study of Lamont and Martin (2003), other genes essential for PVD production in $P. aeruginosa$ PAO1 are identified. Based on their location in the genome, the homologs func-

### Table 3
**Determination of the concentration of fresh and heated PVD by $P. aeruginosa$ PS10.**

| Type of PVD | PVD production OD 405 nm | Conc. (μM) |
|-------------|--------------------------|------------|
| Fresh (Toxin) | 0.524 ± 0.006 | 37.4       |
| Heated (Toxoid) | 0.214 ± 0.002 | 15.3       |

In vivo toxicity of the fresh and heated PVD produced by $P. aeruginos PS10$.

| Type of PVD | PVD conc. (μM) | Mice groups | Injected PVD (ml) | Mortality of mice after days |
|-------------|----------------|-------------|-------------------|-----------------------------|
| Fresh (Toxin) | 37.4 | A | 0.5 | 0/4 0 1/4 25 2/4 50 3/4 75 4/4 100 |
| B | 0.3 |
| C | 0.1 |
| D | 0.5 |
| E | 0.3 |
| F | 0.1 |
| Heated (Toxoid) | 15.3 | A | 0.5 | 0/4 0 0/4 0 0/4 0 1/4 0 2/4 50 |
| B | 0.3 |
| C | 0.1 |

Fig. 2. Phylogenetic tree represented the DNA similarities of the obtained 16S rDNA sequences of Pseudomonas aeruginosa.
tion in other bacteria, and whether or not their expression was PVDs-dependent, 14 potential genes were selected from the PAO1 genome sequence. Eight novel PVD genes have been discovered.

From the previous studies of biofilm formation and PVD production by the 10 MDR P. aeruginosa isolates, it was concluded that the more biofilm formation, the more PVD production, and vice versa.

The association between biofilm production and pathogenicity, and also the association between biofilm production and pyoverdine, were investigated. According to the investigations, biofilm generation is required for pyoverdine synthesis (Visaggio et al., 2015; Kang and Kirienko, 2017; Kang and Kirienko, 2018), which noticed that pyoverdine production and pathogenicity were found to have a robust, and a statistically significant positive association.

It was indicated that all P. aeruginosa isolates (100%) were positive for the lasR QS gene by PCR. The synthetic amplicons were 725 bp for the lasR QS gene. The amplification of the lasR gene doesn’t necessarily indicate lasR function. These consequences were agreed to study conducted by Aghamollaei et al., (2015). The lasR, lasG, and gyrB genes were amplified by uniplex and triplex PCR from the genomic DNA of standard P. aeruginosa. The produced amplicons were 600 bp for lasR, 700 bp for lasG, and 222 bp for gyrB genes.

Moreover, the genomic DNA of S. aureus, E. coli, K. pneumoniae, S. typhimurium, and non-P. aeruginosa species were employed as DNA templates in the same study to determine the specificity of the primers in PCR experiments. Because no amplicons were formed when the genome of another species was employed, the findings indicated that the recommended primers were distinct to P. aeruginosa genes.

Many studies support the role of the P. aeruginosa las QS in biofilm formation (Bala et al., 2011). The lasR and lasG genes specifically recognized the P. aeruginosa strain, according to BLAST data. It should be noticed that quorum sensing systems in Pseudomonas species are equivalent, whereas the QS genes in non-P. aeruginosa species are distinct and conserved (Venturi, 2006). This idea was supported by (Senturk et al., 2012), who investigated virulence factors that were QS-dependent in 82P. aeruginosa urinary tract strains. They found that 78% produced biofilm and QS proficient, demonstrating the importance of QS in P. aeruginosa virulence.

Also, the same idea was concluded by (Mahmoud et al., 2021), who screened 50 clinical P. aeruginosa isolates for biofilm production by the CRA and TCP. It was found that a total of 44 isolates were non-producer biofilm, and only six isolates produced biofilm. PCR analysis of these six isolates for the presence of the lasR QS gene was indicated that all had contained this gene. Out of the remaining 44 biofilm deficient isolates, 30 isolates were lasR QS gene-deficient, indicating a significant relationship between this gene and biofilm formation.

P. aeruginosa can form biofilms, which are cell populations that are encased in a self-secreted extracellular matrix that defends the cells from antibiotics and the host’s immune system (Cassin and Tseng, 2019), resulting in the recalcitrant of bacteria to the antimicrobial medication alongside the host’s immunological defenses (Pestak et al., 2019).

In the present study, the injection of mice by the fresh PVD caused 100% mortality within five days by 0.3 ml of its concentration (37.4 μM). In contrast, 0.3 ml (15.3 μM) of heated PVD at 115°C for 20 min (toxoid) caused 50% mortality.

The increasing frequency of drug-resistant bacteria necessitates the investigation of novel therapeutic approaches for the infections of P. aeruginosa. The development of innovative medicines to antagonize pathogen virulence factors as biofilm or toxin generation is the most intensively investigated alternative (Schütze et al., 2015). The heat stability of siderophores produced by Streptomyces mirabilis P16B-1 in the cell-free culture supernatant was examined, and it was discovered that 93.4% of the baseline siderophore content was identifiable following heat treatment.

Intraperitoneal (IP) injections of 0.5, 0.3, and 0.1 ml of fresh hemolysin toxin (purified from Pseudomonas aeruginosa PA01) resulted in 50%, 75%, and 100% mortality in the injected mice, respectively. While the hemolysin lost some of its toxicity after being heated for 15 min in boiling water at doses of 0.5 and 0.3 ml, the mice died at a rate of 50% and 25%, consecutively. The mice survived a 0.1 ml injection of heated hemolysin intraperitoneally (IP) (Allam et al., 2020).

Goss et al. (2018) and Kang et al. (2019), demonstrated that gallium efficiently cures P. aeruginosa infections in mice. Gallium (III) nitrate has been shown to affect bacterial iron homeostasis, which inhibits pathogen development and biofilm formation in P. aeruginosa. Gallium (III) is chelated by the pyochelin and pyoverdine siderophores of P. aeruginosa. Kang et al. (2021), noticed that per the findings, even when the pyoverdine inhibitor 5-fluorocytosine indirectly inhibits bacterial growth in the exitance of gallium, it mostly acts as an anti-virulent, and there is minimal selective pressure for resistance. They also discovered that tetracycline suppresses pyoverdine at doses less than those required to keep bacteria at a distant, allowing it to work in tandem with gallium to suppress the growth of bacteria and save Caenorhabditis elegans throughout P. aeruginosa pathogenesis.

Fluoropyrimidines (such as 5-fluorocytosine, 5-fluorouracil, and 5-fluorouridine) have been demonstrated to suppress pyoverdine production in C. elegans and mice, enhancing survival. C. elegans were rescued with 50 μ M 5-fluorocytosine or 10 μ M 5-fluorouridine, which is compatible with a study by Kirienko et al. (2016) and Kang et al. (2019).

Cysteamine prevents the synthesis of pyocyanin and pyoverdine, the fluorescent, and iron-chelating pyoverdine virulence factor (Fraser-Pitt et al., 2021). P. aeruginosa PA01 type strain, NHH57388A alginate-producing strain, and PA14 clinical isolates were cultured in MHB, and the impact of various cysteamine dosages on pyocyanin secretion was measured. They discovered that 2, 20, 100, and 200 mg/L cysteamine inhibited pyoverdine fluorescence and greatly diminished the toxicity of NHH57388A in C. mellonella sterile-filtered supernatants, as well as the fast melanization, was observed 1 h after injection.

5. Conclusion

High biofilm and PVD forming are a significant virulence with presence of lasR gene in MDRPA clinical isolates. It was reported that the newest therapeutic strategies inhibit pyoverdine’s function by toxin neutralization of PVD by heating to form toxoid PVD, which can create high protection and host immune defense in mice. Finally, we concluded that it is necessary to use anti-virulence strategies as a potential direction for a new technique for mitigating the spread of virulent infections and resistance.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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