



Original Article

Beta-lactamase-producing *Pseudomonas aeruginosa*: Phenotypic characteristics and molecular identification of virulence genes

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Abstract

Background: *Pseudomonas aeruginosa* causes common infections in immunocompromised and cystic fibrosis patients. However, drug resistance capability and release of virulence factors play a key role in bacterial pathogenicity.

Methods: Beta-lactamase-producing clinical isolates of *P. aeruginosa* were screened for biofilm formation and pigment production. Subsequently, all the isolates were subjected to the detection of six virulence genes (*OprI*, *OprL*, *LasB*, *PlcH*, *ExoS*, and *ToxA*).

Results: Among beta-lactamase-producing isolates ($n = 54$), about 85.18% ($n = 46$) were biofilm producers. Pigment production was observed in 92.59% ($n = 50$) isolates. Clinical samples were subsequently screened for detection of virulence factors. Among them, 40.74% ($n = 22$) isolates were found to be *OprI* positive, while 29.62% ($n = 16$) were *OprL* producers. In the case of *LasB* and *PlcH*, 24% ($n = 13$) and 18.5% ($n = 10$) isolates produced these virulence genes, respectively. Among the isolates, 37.03% ($n = 20$) and 33.33% ($n = 18$) expressed virulence factors *ExoS* and *ToxA*, respectively. Furthermore, 42.59% ($n = 23$) isolates coproduced more than one type of virulence factors.

Conclusion: In the current study, pigment display, biofilm formation, and virulence genes were detected in *P. aeruginosa* clinical isolates. Such factors could be crucial in the development of drug resistance.

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Keywords: beta-lactamases; *Pseudomonas aeruginosa*; virulence

1. Introduction

Pseudomonas aeruginosa infections are prevalent in immunocompromised and cystic fibrosis patients, causing numerous acute and chronic infections.¹ These pathogens are capable of surviving in different environmental conditions by utilizing their diverse metabolic and virulence patterns.² *P. aeruginosa* is capable of colonizing the respiratory tract in spite of its different ecological origin.³ Virulence genes have a different level of

intrinsic expression, which leads to a variable level of pathogenicity in infected individuals.⁴ Mainly, these infections arise due to development of drug resistance patterns, biofilm formation, and virulence factor production.² Biofilm, a source of chronic and persistent infection, presents strong resistance to the immune system and antibiotics.⁵ Growth of *P. aeruginosa* in varying environments can enhance pigment production.⁶ Their outer membrane proteins (*OprI* and *OprL*) activated the immune system of a patient and emerged as a strong candidate for *P. aeruginosa* vaccine development.^{7,8} Additionally, fluctuation in environmental conditions can enhance elastase expression in these pathogens.⁶ *P. aeruginosa* is capable of producing two types of phospholipase, of which the hemolytic type (*PlcH*) hydrolyzes sphingomyelin along with phosphatidylcholine.⁹ Virulence factor *ToxA* was reported from patients having lung infection, while *ExoS* is more prevalent in cystic fibrosis

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patients.^{3,10} The expression of cellular and extracellular virulence factors of *P. aeruginosa* is regulated via cell signaling pathways.¹¹ The strong correlation between virulence genes and source of infection can help control these infections in the community.⁴ The most effective mechanism that regulates their expression was quorum sensing, and therefore targeting these key regulators will improve therapeutic success in the future.² The present study documented the extent of pigment production, biofilm formation, and presence of virulence factors in beta-lactamase-producing *P. aeruginosa*. To the best of our knowledge, this is the first report to reveal the status of *P. aeruginosa* virulence factors in Pakistan.

2. Methods

Beta-lactamase-producing *P. aeruginosa* ($n = 54$) isolates were screened in randomly collected samples from pus, urine, blood, sputum, and wounds. Both phenotypic and molecular methods were used to identify beta-lactamase-producing *P. aeruginosa* (data not shown). All beta-lactamase-producing isolates were further processed for evaluation of pigment production and biofilm formation. Subsequently, these clinical isolates were subjected to the detection of six virulence genes (*OprI*, *OprL*, *LasB*, *PlcH*, *ExoS*, and *ToxA*) using polymerase chain reaction (PCR).

2.1. Pigment production

Pigment production was evaluated on cetrinide agar, which is used as a selective culture media for *P. aeruginosa*. Colonies that appeared yellow brown in color were characterized as pyoverdinin, and those that were blue in appearance were considered to be pyocyanin producers.

2.2. Biofilm screening

Biofilm production was evaluated by the tube method as previously described.¹² Briefly, after 18–24 hours of incubation bacterial cultures were decanted and washed with freshly prepared phosphate buffer saline. Crystal violet (0.1%) was

applied to visualize any type of biofilm formation in the test tube. Biofilms were classified as strong, moderate, and weak on the basis of the specific biofilm patterns that appeared in the test tubes, and those having no visible material were considered as negative.¹²

2.3. DNA extraction and virulence gene detection

DNA was extracted from all isolates using a protocol as described previously.¹³ PCR was performed to detect all selected genes using primers listed in Table 1.³ PCR mixture was obtained from Gene Link, Hawthorne, NY, USA, and all reactions were conducted under proper conditions: initial denaturation at 94°C for 4 minutes, followed by 35 cycles of 94°C for 45 seconds, polymerization at 72°C for 1 minute, and final extension for 5 minutes and 1 minute for annealing as noted in Table 1. Amplified products of all virulence genes were further analyzed using ethidium bromide-mixed agarose gel (1.5%) under a UV transilluminator.

3. Results

Beta-lactamase-producing isolates ($n = 54$) were further analyzed for phenotypic and virulence gene profiles. *P. aeruginosa* produced characteristic pigments in the form of pyoverdinin or pyocyanin. A total of 92.59% ($n = 50$) isolates produced these pigments. Among pigment-producing isolates, 64% ($n = 32$) demonstrated pyoverdinin and 36% ($n = 18$) exhibited pyocyanin, as shown in Table 2.

All isolates were further evaluated for biofilm production using the tube method. Ultimately, 85.18% ($n = 46$) isolates produced biofilms, which were classified as strong, moderate, and weak on the basis of their appearance. Among biofilm-producing isolates, 47.88% ($n = 22$) produced strong, 30.43% ($n = 14$) moderate, and 21.73% ($n = 10$) weak biofilm patterns. Isolates that had not produced an observable amount of biofilm were classified as nonproducers and their strength was 17.39% ($n = 8$), as demonstrated in Table 2.

Virulence genes were investigated among all beta-lactamase-producing isolates using PCR. There were 54%

Table 1
List of primers used.

Primers used	Target gene	Sequence of primers 5' → 3'	Annealing temperature (°C)	Fragment size (bp)	Reference no.
ToxA-F	<i>ToxA</i>	GGTAACCAGCTCAGCCACAT	58	352	10
ToxA-R		TGATGTCCAGGTCATGCTTC			
ExoS-F	<i>ExoS</i>	CTTGAAGGGACTCGACAAGG	55	504	
ExoS-R		TTCAGGTCCGCGTAGTGAAT			
LasB-F	<i>LasB</i>	GGAATGAACGAAGCGTTCTC	56	300	
LasB-R		GGTCCAGTAGTAGCGTTGG			
PlcH-F	<i>PlcH</i>	GAAGCCATGGGCTACTTCAA	56	307	
PlcH-R		AGAGTGACGAGGAGCGGTAG			
OprL-F	<i>OprL</i>	ATGGAAATGCTGAAATTCGGC	57	504	21
OprL-R		CTTCTCAGTCTCGACGCGACG			
OprI-F	<i>OprI</i>	ATGAACAACGTTCTGAAATCTCTGCT	57	249	
OprI-R		CTTGCGGCTGGCTTTTCCAG			

Table 2
Phenotypic characteristics and virulence gene profiles of beta-lactamases producing *P. aeruginosa*.

Isolate no.	Pigment production		Biofilm pattern			<i>OprI</i>	<i>OprL</i>	<i>LasB</i>	<i>PlcH</i>	<i>ExoS</i>	<i>ToxA</i>
	Pyoverdinin	Pyocyanin	Strong	Moderate	Weak						
PA.1	+		+			-	-	-	-	+	-
PA.2		+		+		-	-	-	-	+	-
PA.8	+		+			-	-	-	-	-	+
PA.10	+		+			-	-	-	-	+	-
PA.11	+				+	-	-	-	-	+	-
PA.12	-	-		+		-	-	-	-	+	-
PA.15	+				+	-	-	-	+	-	+
PA.16	+		+			+	-	-	-	-	-
PA.20	+			+		-	-	+	-	+	-
PA.21	+			+		-	-	-	-	+	-
PA.22	+		+			+	+	-	-	-	+
PA.23		+	-	-	-	-	+	-	-	+	+
PA.24	+		+			-	-	-	-	+	-
PA.26	+		+			-	-	-	-	+	-
PA.28	+				+	+	-	-	-	+	-
PA.30		+			+	-	+	-	-	-	-
PA.33	-	-	+			+	+	-	-	-	-
PA.34	+			+		-	-	-	+	-	-
PA.35		+			+	+	-	-	-	-	-
PA.37		+			+	-	-	+	-	-	-
PA.38	+			+		+	+	-	-	+	+
PA.39	+		-	-	-	-	-	+	-	-	+
PA.41	+		+			-	-	-	-	+	-
PA.43	+		-	-	-	-	-	-	-	-	+
PA.44		+	+			+	-	-	+	-	-
PA.48		+		+		-	+	-	+	-	+
PA.49		+			+	+	-	-	+	-	-
PA.50	+		-	-	-	+	-	-	-	-	+
PA.51		+			+	+	-	-	-	-	-
PA.52	-	-		+		-	-	-	-	+	-
PA.53	-	-		+		+	-	+	-	-	-
PA.56	+		+			+	+	+	-	-	-
PA.57		+	+			-	-	+	-	+	+
PA.58	+			+		-	-	-	+	-	+
PA.62		+			+	-	-	+	-	-	-
PA.65	+		-	-	-	-	+	-	-	-	-
PA.66	+		+			+	+	-	-	+	-
PA.67		+	-	-	-	+	-	-	-	-	-
PA.68	+		+			-	-	-	-	+	-
PA.69	+		+			-	+	+	-	-	-
PA.70	+			+		-	-	-	-	-	+
PA.73	+				+	+	+	+	-	+	+
PA.75	+		+			-	-	-	-	+	-
PA.76	+		+			+	-	+	-	-	+
PA.83	+			+		-	-	-	-	-	+
PA.84		+	+			+	+	-	-	-	-
PA.85		+	-	-	-	-	-	+	-	-	-
PA.88		+	+			+	+	+	+	-	+
PA.91	+			+		-	+	-	-	-	-
PA.92		+	-	-	-	-	-	-	+	-	-
PA.94		+	+			+	+	-	-	-	+
PA.100	+		+			+	+	+	+	-	-
PA.101	+			+		+	-	-	-	-	-
PA.102		+	+			+	-	-	+	+	+
Total (54)	32	18	22	14	10	22	16	13	10	20	18

($n = 54$) isolates that had one or more than one type of all these six virulence factors. Among all the isolates, 40.74% ($n = 22$) produced *OprI* (249 bp in size) and 29.62% ($n = 16$) expressed *OprL* (504 bp) virulence genes. Furthermore, these isolates were evaluated for *LasB* (300 bp) and *PlcH* (307 bp)

virulence factors, and 24% ($n = 13$) and 18.5% ($n = 10$), respectively, were found to be positive for these factors (Fig. 1 and Table 2). Subsequently, these isolates were screened for *ExoS* and *ToxA*, having a product size of 504 bp and 352 bp, respectively. The prevalence of *ToxA* isolates was 33.33%

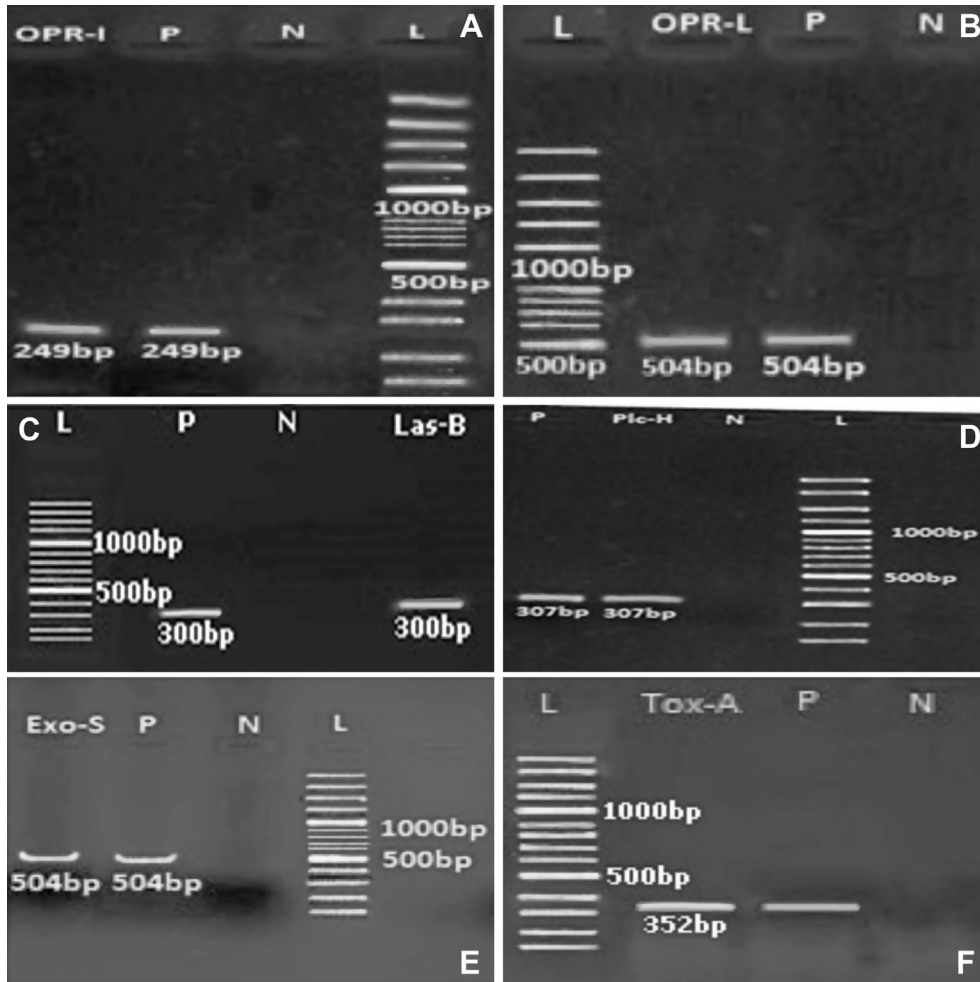


Fig. 1. Amplification products of virulence genes: (A) *OPRI* (249 bp), (B) *OPRL* (504 bp), (C) *LasB* (300 bp), (D) *PlcH* (307 bp), (E) *ExoS* (504 bp), and (F) *ToxA* (352 bp).

($n = 18$); *ExoS* had a higher prevalence of 37.03% ($n = 20$) compared with *ToxA*.

Coproduction of virulence factors was observed among all these isolates. A total of 42.59% ($n = 23$) isolates produced more than one type of virulence factors. Among these isolates, 13 (24%) were determined to be positive for two virulence factors, five (9.25%) produced three virulence factors, four (7.4%) produced four virulence factors, and one expressed five virulence factors.

4. Discussion

P. aeruginosa, one of the major human opportunistic pathogens secreting various toxins and involved in different types of infections, is considered to be a primary contributor in the pathogenesis of infected individuals.² Production of virulence factors was analyzed in extended spectrum beta lactamases (ESBL)-producing *P. aeruginosa*, and it was established that a strong correlation exists between secretion of toxins and beta-lactamase producers.¹⁴ It was revealed that pigment production was more significantly associated with drug resistance than the production of virulence factors such as

elastases and proteases in clinical isolates of *P. aeruginosa*.¹⁵ In another study, pigment production was consistent with our results.¹⁴ Biofilm formation was compared with previously reported cases of clinical isolates, and it was noted that the prevalence of biofilm formation was consistent with our study results, which implied that biofilm formation has a strong correlation with the drug resistance pattern.¹⁶ Biofilm formation can strengthen ESBL-carrying bacteria more pathogenically compared with non-ESBL producers, as in our case, where all were biofilm producers.¹⁷ There has been some measure of phenotypic diversity linked with biofilm formation which leads to mortality and morbidity. Therefore, exploration of the mechanistic details of biofilm formation will open up new therapeutic venues to control these pathogens.¹⁸

Distribution of virulence factor *ExoS* was higher compared with the data shown in another study previously. The *ToxA* prevalence was lower in our study than that in a previous study. Expression of virulence factors can be retarded using azithromycin, which inhibits autoinducers that are involved in the expression of these genes.¹⁹ Overall, expression levels of both *ExoS* and *ToxA* were lower in our study as compared with those in a previous study.⁴ When

ExoS acts as a mitogen, it can promote activation of T lymphocytes leading to inflammation, which is the cause of pathogenesis in cystic fibrosis patients.²⁰ An elevated incidence of ExoS was reported in drug resistance isolates in a study conducted in Iran.²¹ The expression of these virulence factors is required to cause infection in patients, particularly in those having pulmonary infection.²²

LasB incidence was slightly higher in our isolates as compared with cases reported in another study, which involved the detection of virulence genes in pigment- and nonpigment-producing *P. aeruginosa*.¹⁵ ESBL-harboring bacteria have more LasB activity than non-ESBL-harboring bacteria, which highlights their strong interaction with the drug resistance pattern.²³ Another study disclosed the occurrence of LasB and PlcH virulence factors in clinical isolates of *P. aeruginosa*, where the prevalence of both virulence factors was higher in contrast to our findings.²⁴ Some inhibitors having interactions with quorum-sensing receptors such as LasR and RhlR can affect the pathogenicity of *P. aeruginosa* in humans, and it will be helpful in developing new therapeutic strategies in the future for patients having cystic fibrosis infections.²⁵ Expression of virulence genes both in cystic and in noncystic fibrosis revealed that it might not have a strong link with the type of infection, but can have an impact on treatment strategies.³

In conclusion, beta-lactamase-harboring bacteria exhibit a strong drug resistance pattern against different groups of antibiotics. Besides the production of resistance-related enzymes, these bacteria release pigments and also express different virulence genes that might be the cause of development of the resistance pattern in *P. aeruginosa*. Further studies will be helpful in revealing the correlation between the drug resistance pattern and virulence factor expression.

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