Research Paper Medical Science

Detection of Pel A Gene in P. aeruginosa from Clinical Samples Using Polymerase Chain Reaction with Reference to Biofilm Production In N.E India.

Indu Sharma Microbiology department, Assam University, Silchar-788011, India.

Daijee Choudhury Microbiology department, Assam University, Silchar-788011, India.

ABSTRACT

Objectives: The aims of the current study were to determine the biofilm formation in P. aeruginosa clinical isolates and to evaluate the role of the selected PelA gene in biofilm formation using PCR method in N.E India.
Methods: A total of 45 clinical isolates were isolated from Silchar medical college and hospital, Silchar, Assam followed by antibiotic susceptibility test for all the strong, intermediate, weak and non-biofilm producing strains against aminoglycosides and beta lactam antibiotics. Using biofilm assays, the ability of P. aeruginosa isolates to form biofilm was estimated and according to the OD values, isolates was classified into weak, moderate or strong biofilm producers. A PCR assay was carried out for detection of PelA gene which was involved in formation of biofilm among the clinical isolates.
Results: Of the 47 isolates, 42(89.3%) isolates confirmed the production of biofilm amongst which 34 (72.34%) were strong, 5 (10.63%) intermediate and 3(7.14%) weak biofilm producers. The aminoglycosides set of antibiotics represented 78.5% resistant towards gentamycin, 83.3 % Tobramycin, 80.9% Amikacin and finally 73.8 % towards Kanamycin. The beta lactam antibiotics exhibited the resistant pattern higher in biofilm producing strains where ceftazidime expressed 69.0% resistance, cefepime 61.9%, cefixime 66.6% and piperacilin 57.1%. Further, in the present study PelA gene was expressed heavily (80%) amongst the biofilm producing isolates and those associated with the polysaccharide stage of biofilm development and maintenance (97%). It was also observed that the phenotypically positive isolates for production of biofilms in vitro were genotypically positive for expression of PelA gene while those isolates phenotypically producing biofilm and harbouring PelA gene were also found to be resistant towards aminoglycosides and beta lactam antibiotics.
Conclusions: The present study showed that most of the biofilm producing strains were highly resistant to even higher generations of aminoglycosides and beta lactam group of antibiotics, therefore, more work is needed to fully elucidate this antibiotic resistance mechanism in biofilms and develop new therapeutic strategies.

KEYWORDS Biofilm, PelA gene, antibiotic resistance and PCR

Introduction

P. aeruginosa one of the most studied microbes in the context of biofilms (6,11,5), yet the genetic basis for the formation of extracellular matrices in different *P. aeruginosa* biofilms remains poorly defined. There are strong indications that this organism forms multicellular aggregates within sites of infection, e.g. in the lungs of cystic fibrosis patients or on the surfaces of contaminated catheters (8,17). Biofilm is an aggregate of microorganisms in which cells are stuck to each other on to a surface and produce matrix of extracellular polymeric substance. Biofilm formation is regulated by the expression of polysaccharide intracellular adhesion molecule, which mediates cell to cell adhesion and is a product of different genes (26).

The familiar mechanisms of antibiotic resistance, such as efflux pumps, modifying enzymes, and target mutations (27) do not seem to be responsible for the protection of bacteria in a biofilm. Even sensitive bacteria that do not have a known genetic basis for resistance can have profoundly reduced susceptibility when they form a biofilm (21). Bacteria that lack protective mutations or that lack plasmids or other mobile genetic elements carrying resistance genes nevertheless become less susceptible when grown in the biofilm state (2). Antibiotic sensitivity is usually quickly restored when bacteria are dispersed from a biofilm. The rapid reversal of resistance upon dispersion from a biofilm suggests an adaptive resistance mechanism rather than a genetic alteration (23). Many different types of antibiotics have been tested against bacterial biofilms, and in almost every case bacteria in the biofilm mode of growth are found to be less sensitive to killing than the same strain when grown in free aqueous suspension. These observations tell us that the capacity to form protective biofilms is widely distributed among microbes and that the resistance mechanisms that operate in biofilms constitute a broad spectrum defence (23).

Hence, the present study concentrates on biofilm formation against biofilm and non-biofilm producers along with their antibiotic profile and the effect of *PelA* gene on biofilm formation in *P. aeruginosa* isolated from clinical isolates.

Materials and methods

Clinical isolates and phenotypic identification

A total of forty-seven isolates were recovered from different clinical samples by isolation on Mackonkey agar (Hi-Media) and cetrimide agar media (Oxoid). Isolates were also cultured aerobically on tryptic soya agar (Hi-Media). All the clinical samples collected were from silchar medical college and hospital (SMCH) and from different community areas, North-East India.

The distinct isolated colonies were identified on the basis of their morphological and biochemical characters. The shape and colours of the colonies were examined under the microscope after Gram staining. Isolates were biochemically confirmed for the activities of oxidase, catalase, methyl red, voges-prauskauer test, urease, motility, indole production, citrate utilization, gelatinase and sugar fermentation tests through a series of conventional biochemical tests.

Biofilm assay

Phenotypic screening of the biofilms produced by the isolates was performed *in-vitro* by tube, Congo red and slide methods as previously described (20) with slight modifications. Experiments were performed in triplicates and OD for each isolate was calculated. The OD values were considered as an index of

bacteria adhering to surface and forming biofilms.

Antimicrobial susceptibility testing
Antibiotic susceptibility of the isolates was assayed according to Kirby-Bauer disc diffusion method (3) on Mueller-Hinton agar (MHA). The isolates were freshly inoculated on saline water for detection of turbidity via comparison with 0.5Mc. Farland solution (1.5x108 CFU/ml). The antibiotic disks used were of HI-MEDIA Laboratories, Mumbai, India, consisting of the following: Cefixime (30µg), Ceftazidime (30µg), Cefepime (20µg), Piperacilin (10µg), Gentamycin (10µg), Amikacin (5µg), Tobramycin (10µg) and Kanamycin (10µg). Positive tests were indicated by zones of inhibition which were measured by using the zone size interpretative tables provided by the manufacturer of the discs.

Genotypic identification
DNA Extraction
DNA of *P. aeruginosa* isolates was extracted and purified using Genomic DNA Mini Kit (blood/cultured cell).

Primer selection
The primers (Forward and Reverse) used in PCR were specific for *PelA* gene which was chosen according to Colvin *et al*., 2011. The sequence of primer used in this study is presented in Table (1).

Table1: Sequences of primers for *PelA* gene

Gene		Nucleotide sequences (5′ 3′)	Products bp	Reference
PelA	F	CCTTCAGCCATCCGTTCTTCT	118bp	Colvin *et al*., 2011
	R	TCGCGTACGAAGTCGACCTT		

PCR amplification
PCR for amplification of *PelA* gene was performed using 12.5µl of Tag Green Master Mix 2x DNA polymerase (22), specific primers for *PelA* gene (1µl forward and 1µl reverse), 2µl of *P. aeruginosa* DNA extract as a template, 8.5µl of nuclease free water to make the final reaction volume up to 25µl. The PCR conditions started with thermocycler program that is initial denaturation at 95°C for 2 minutes, 34 cycles of denaturation at 95°C for 15 seconds, 45°C for 1 minute, and 72°C for 45seconds and final extension at 72°C for 7 minutes.

Agarose gel electrophoresis
Five microliters of the reaction mixture and 1µl marker DNA (100bp DNA ladder mix; MBI Fermentas, USA) mixed separately with 1µl of 6X gel loading dye (MBI Fermentas, USA) and were analyzed by submarine gel electrophoresis in 1% agarose (Hi-Media, Mumbai, India) at 60 V for 1 hour and 20 min. or until the second dye marker had run 3/4th of the gel (25). The reaction products were visualized with UV light after staining with ethidium bromide. The identities of the amplicons were confirmed by comparison of the amplicon sizes with the predicted sizes and photographed (22).

Results
All the 47 isolates had a growth on cetrimide agarmedium and MacConkey agar medium, were oxidase and catalase positive and exhibited no changes in the colour of the medium. 100% motility was observed in all the isolates; indole, methyl-red and voges-Proskauer tests were observed as negative; all of them represented citrate utilization, gelatinase activity and urease test as positive, were non-lactose fermenters on MacConkey agar medium and non-fermentive on TSI. All the 47 isolates were identified as *P. aeruginosa* (10,28).

Antibiotic susceptibility was tested for all the strong, intermediate, weak and non-biofilm producing strains which were observed against commercially available aminoglycosides and beta lactam antibiotics. Aminoglycosides antibiotics represented 78.5% resistant to gentamycin, 83.3 % to Tobramycin, 80.9% to Amikacin and 73.8 % to Kanamycin. In the present investigation, it was observed that the resistant rate was higher as compared to susceptible percentage for biofilm producers. Furthermore, the beta lactam antibiotics presented the resistant pattern as ceftazidime (69.0%), cefepime (61.9%), cefixime (66.6%) and piperacilin (57.1%) which also clearly indicates that the resistant pattern was also higher in case of beta lactam antibiotics in biofilm producing strains. The antibiotics when combined with a β-lactam inhibitor, mainly cefoperazone/sulbactam and piperacillin/sulbactam, exhibited the resistance rates of 40.4% and 38.2%, respectively.

Using biofilm assays, the ability of *P. aeruginosa* isolates to form biofilm was estimated. According to the OD values, biofilm production of isolates was classified into weak, moderate or strong biofilm producers (Table 2).

Table: 2 Biofilm formations of *P. aeruginosa* isolates (using tube, Congo red and slide assays)

Strong biofilm producers	Intermediate biofilm producers	Weak biofilm producers	Non-biofilm producers	Total
34(72.34%)	5(10.63%)	3(7.14%)	5(10.63%)	47

PCR was carried out for detection of *PelA* gene which was involved in formation of biofilm among the clinical isolates and was expressed heavily in 80% of the isolates. This was importantly associated to the polysaccharide stage of biofilm development and maintenance and all the isolates harbouring *PelA* gene were also phenotypically positive for biofilm formation.

Fig: 1. Agarose gel electrophoresis of *PelA* gene. DNA ladder used was 100bp. Lanes 1, 3, 4 and 5 exhibits the amplified *PelA* gene (118 bp). Lane 2 is negative control.

Discussion
In the present study, *P. aeruginosa* was one of the main causes of nosocomial infections exhibiting an intrinsic resistance to several antimicrobial agents (12), biofilm forming and dominant pulmonary pathogen (13). Earlier reports have shown that the antibiotic resistance of bacteria due to biofilm formation contributes to the persistence of bacterial cells and causes problems in the complete eradication of infection (1).

In this study, a total of 47 tested isolates were screened for formation of biofilm phenotypically, of which 42(89.3%) isolates were confirmed for production of biofilm; 34 (72.34%) were strong, 5 (10.63%) were intermediate and 3(7.14%) were weak biofilm producers (15). Isolates from ear swabs and throat swabs were more prone to produce strong biofilm. Further, the isolates producing biofilm from urine samples were poor biofilm producers when compared to ear and throat swabs (7).

Biofilms those produced by tube method expressed a visible film lining the wall and bottom of the tube while the non-biofilm producing strains were unable to form the lining in the tube (26,9). Such *in vitro* production of biofilm may be due to presence of *PelA* gene in the clinical isolates (9) which did not allow the formation of biofilm due to presence of *Pel* mutant strains; whereas biofilms were observed in wild type strains and alginate mutant strains thus revealing that *Pel* is an essential exopolysaccharide which is often required in biofilm matrix formation.

Congo red agar method revealed the presence of strong biofilm producers by exhibiting black colour crystalline colonies while the intermediate biofilm producers produced non-crys-

talline black coloured colonies. Weak or non-biofilm producers exhibited pink-yellow coloured colony morphology in the present study (26,18). The slide method exhibited those biofilm formations that were allowed to grow on glass slides and produced water channel like pores on the glass slides. These pores were more prominent after 96 hours of incubation (7) and the maturity of biofilm matrix was proportional to the increasing incubation period. The slime layers formed in the slide were also stickier after 96 hours incubation in comparison to 24 hours incubation (16). The present study also detected the presence of small voids and channels separating the micro colonies within the pneumococcal biofilm.

The antibiotic susceptibility test was performed for aminoglycosides and beta lactam antibiotics with all the 47 isolated isolates. The aminoglycosides set of antibiotics represented 78.5% resistant towards gentamycin, 83.3 % towards Tobramycin, 80.9% towards Amikacin and finally 73.8 % towards Kanamycin. From the above Tobramycin in comparison to other aminoglycosides exhibited increased levels of resistance which may be due to presence of *PelA* gene (19,14). Colvin et al., in 2011 hypothesized that *Pel* is capable of providing protection to planktonic cells when artificially overexpressed, thus suggesting that *Pel* plays an important protective role in biofilms of pseudomonas strains. The beta lactam antibiotics represented the resistant pattern higher in biofilm producing strains. Ceftazidime expressed 69.0% resistance, cefepime 61.9%, cefixime 66.6% and piperacilin 57.1%. A similar report was also observed where beta lactam antibiotic resistance rate was higher than their susceptibility due to production of beta lactamase enzymes in some isolates of pseudomonas (24).

In the present study, the isolates producing strong, intermediate and weak biofilms were screened for *PelA* gene which is responsible for production of biofilm. *Pel* is crucial for maintaining cell to cell interactions in a PA14 biofilm, serving as a primary structural scaffold for the community. It also plays a secondary role by enhancing resistance to aminoglycoside antibiotics and this protection occurs only in biofilm populations (4).

This study observed that *PelA* gene was expressed heavily (80%) amongst the biofilm producing isolates and those associated with the polysaccharide stage of biofilm development and maintenance (97%) (22). The phenotypically positive isolates for production of biofilms *in vitro* were genotypically positive for expression of *PelA* gene while those isolates phenotypically producing biofilm and harbouring *PelA* gene were also found to be resistant towards aminoglycosides and beta lactam antibiotics (4,15)

Conclusion

Bacteria that have the ability to form biofilms, coupled with the emergence of multidrug resistant strains, are significantly an increasing concern in healthcare. The present study showed that most of the biofilm producing strains were highly resistant to even higher generations of aminoglycosides and beta lactam group of antibiotics. It was also observed that most of the isolates showing resistance to aminoglycosides antibiotic carried *PelA* gene. Though biofilm producing strains showed resistance to aminoglycosides and beta lactam antibiotics, the presence of other intrinsic resistance mechanisms in biofilm producing *Pseudomonas* sp. cannot be neglected. Henceforth, more work is needed to fully elucidate the antibiotic resistance mechanisms in biofilms and develop new therapeutic strategies.

Acknowledgement

We would like to acknowledge Silchar medical college and hospital for assistance in sample collection. This work was carried out with the support of Microbiology department, Assam University, Silchar, Assam.

Conflict of interest: None

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