

Comparative analytic study of exotoxin A gene production among ESBL and non- ESBL producing clinical isolates of *Pseudomonas aeruginosa*

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Abstract: Exotoxin A is an extracellular enzyme that is produced by most clinical strains of *Pseudomonas aeruginosa* which inactivates and inhibits the protein biosynthesis. The present study aims to detect any possible correlation or any significant association between the production of exotoxin A and antibiotic resistance among *P. aeruginosa* clinical isolates or they can act independently, which might have implications for treatment and control. A Prospective study done on 60 isolates of *Pseudomonas aeruginosa* which included 30 ESBL and 30 Non- ESBL producers were randomly selected from total 203 isolates from various clinical samples in a tertiary care hospital, Puducherry. All isolates were screened for ESBL production using Phenotypic confirmatory disc diffusion test. Detection of Exotoxin A (*toxA*) gene expression were done by conventional PCR. Out of 60 clinical isolates of *Pseudomonas aeruginosa* showed 70% (42/60) positivity for exotoxin A by PCR. Among 30 ESBL positive isolates, 76.7% (23/30) were positive and among 30 ESBL negative isolates 63.3% (19/30) were positive for *toxA* gene production. In our study, 70% positivity of *toxA* gene in clinical isolates of *Pseudomonas aeruginosa* were obtained of which 76.7% were from ESBL and 63.3% were from non-ESBL producers. Henceforth, the association between exotoxin A gene expression and resistance mechanism like ESBL production was not statistically significant in our study, which may indicate that there need not be always a direct correlation between virulence gene and antibiotic resistance and they may act independently.

Date of Submission: 10-02-2018

Date of acceptance: 24-02-2018

I. Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen in hospitalized patients. The pathogenicity of *P. aeruginosa* is contributed by several virulence factors such as exotoxin A, exoenzyme S, elastase and sialidase which are tightly regulated by quorum sensing systems.¹ Of importance is type III secretion system, which secretes exotoxin A and exoenzyme S, which inhibits protein biosynthesis.^{2,3}

Exotoxin A is an extracellular enzyme that catalyzes the transfer of the ADP(Adenosine diphosphate-ribose) moiety from NADP(Nicotinamide-adenine dinucleotide) to elongation factor 2, which results in the inactivation and inhibition of protein synthesis. It is produced by most clinical strains of *Pseudomonas aeruginosa*. This enzyme is a single-chain polypeptide with A and B fragments which mediates enzymatic and cell-binding functions, respectively.⁴

ExoA is toxic to eukaryotic cells and inhibit protein inside the cell and damage to lung tissue and the brain. Like Diphtheriatoxin, it causes the ADP ribosylation of eukaryotic elongation factor 2 resulting in inhibition of protein synthesis in the affected cell and induces the production of interleukin-1.⁵ *Pseudomonas aeruginosa* is the most common opportunistic pathogen in the hospital, hence it is important to detect various resistance mechanism and virulence factor produced by it.⁴

In recent years, *Pseudomonas aeruginosa* infections are difficult to treat due to emergence of newer β -lactamases such as Extended Spectrum β -lactamases (ESBL), AmpC β -lactamases and Carbapenemases. Major mechanisms causing resistance to the β -lactam antibiotics in *Pseudomonas aeruginosa* are the production of β -lactamases, reduced outer membrane permeability and altered affinity of target Penicillin binding proteins.⁶

II. Material And Methods

A Prospective Analytical study was conducted in a tertiary care teaching hospital in Puducherry for duration of one year, which included a total of 203 consecutive non-duplicate isolates of *Pseudomonas*

aeruginosa obtained from various clinical samples like pus (103), urine (50) and sputum (50) sent to laboratory for culture and sensitivity. **Study Design:** Prospective analytical study

Study Location: This study was conducted in a Department of Microbiology, tertiary care teaching hospital in Puducherry

Study Duration: May 2015 to May 2016.

Sample size: 203 isolates of *Pseudomonas aeruginosa*

Subjects & selection method: A total of 203 consecutive non-duplicate isolates of *Pseudomonas aeruginosa* obtained from various clinical samples like pus (103), urine (50) and sputum (50) sent to laboratory for culture and sensitivity

Inclusion criteria:

1. *Pseudomonas aeruginosa* isolated from all clinical samples (pus, urine, sputum)

Exclusion criteria:

1. Repeat isolates of *Pseudomonas aeruginosa* from the same patients.
2. Isolates of *Pseudomonas aeruginosa* from environmental samples.

Procedure methodology

Pseudomonas aeruginosa isolated from those clinical samples were identified and confirmed based on standard laboratory technique.⁷ All isolates of *Pseudomonas aeruginosa* from clinical samples were further screened for susceptibility to the 3rd generation cephalosporins like ceftazidime (30 µg/disk), cefotaxime (30 µg/disk) and ceftriaxone (30 µg/disk) by standard disc diffusion method as recommended by Clinical and Laboratory Standards Institute, 2015 (CLSI).⁸

Isolates which were resistant to at least one of the 3GCs were selected for the study and were processed for ESBLs production. If a zone diameter of ≤ 22 mm for ceftazidime, ≤ 27 mm for cefotaxime and ≤ 25 mm for ceftriaxone were recorded, those isolates are considered positive for ESBL producers and further were confirmed for ESBL production using Phenotypic confirmatory disc diffusion test / Combined disk diffusion method.⁹

Phenotypic confirmatory disc diffusion test (PCDDT)/ Combined disk diffusion method:

Ceftazidime and cefotaxime discs (30 µg) alone and in combination with clavulanic acid (30/10 µg) were applied on Mueller Hinton agar plate, inoculated with the test isolates. Diameter of zone of inhibition was measured after overnight incubation at 37 °C. An increase of ≥ 5 mm in diameter of zone of inhibition of the combination discs in comparison to the cefotaxime or ceftazidime kept alone was considered to be a marker for ESBLs producing isolates.⁸

E. coli ATCC 25922 strain was used for quality control.

Detection of *toxA* gene by Conventional PCR:

A total of 60 isolates were randomly selected from 203 isolates, which included 30 ESBL and 30 Non-ESBL producers, which were analysed for *toxA* gene expression by conventional PCR.

Isolation of genomic DNA

P. aeruginosa was grown overnight in LB broth at 37°C. Bacterial genomic DNA was isolated using EZ10-Spin column bacterial DNA Mini-Preps Kit (Bio Basic Inc., Ontario, Canada) as per the manufacturer's instructions.

Primer selection

The primers used in this study is based on the published sequence,¹⁰ the primer for ETA1 sequence specific to *P. aeruginosa* were chosen with an amplification of -396-bp region of the structural gene.

Region	Size	Primer	Sequence	Tm
1001-1024	396	ETA1	5'-GACAACGCCCTCAGCATCACCAGC-3'	73
1373-1396	396	ETA2	5'-CGCTGGCCCATTCGCTCCAGCGCT-3'	73

Amplifications

The amplification reaction was performed by using a PCR thermal cycler (Bio-Rad) with 2X Taq DNA polymerase Master mix (Ampliqon, Rødovre, Denmark). The reaction mixture (20 µl/reaction total volume) consisted of 6 µl of sterile water, 10 µl of 2x PCR Master Mix containing 1.

5 mM MgCl₂, 1 µl of each primer (stock concentration, 100 µM), 2 µl of template. The samples were subjected to 35 cycles of amplification.

Preincubation was at 95°C for 2 min. 35 PCR cycles were run under the following conditions: denaturation at 94°C for 1 min, primer annealing at 68°C for 1 min, and DNA extension at 72°C for 1 min in each cycle. After the last cycle, the PCR tubes were incubated for 7 min at 72°C. 10 µl of the reaction mixture was analyzed by standard gel electrophoresis (1.5 % µg/ml agarose; 5 V/cm), and the reaction products were visualized by staining with ethidium bromide (0.5 µg/ml in the running buffer). A reagent blank well which contained all components of the reaction mixture minus template DNA,with sterile distilled water was substituted was included in every PCR procedure step.¹¹

Statistical analysis:

- Percentages was calculated for categorical variables.
- Statistical analysis of the results was performed by Pearson’s Chi-square test, and the significance level was set at P < 0.05, using SPSS version 22

III. Result

A total 203 isolates of *Pseudomonas aeruginosa* (103 isolates were from pus, 50 isolates from urine and 50 isolates from sputum) were included in our study. Of 203 isolates, 52 were ESBL and 151 isolates were non – ESBL producers(Table 1)

Table 1: Distribution of β-Lactamase in Clinical Isolates of *Pseudomonas aeruginosa*(n=203)

Sample	ESBL(%)	Non-ESBL(%)
Pus(103)	31(59.6%)	72(47.7%)
Urine(50)	12(23.1%)	38(25.2%)
Sputum(50)	9(17.3%)	41(27.2%)
Total (203)	52(25.6%)	151(74.4%)

In our study, we have included 30 ESBL Producers and 30 non –ESBL producers , a total of 60 clinical isolates of *Pseudomonas aeruginosa*., These 60 isolates were from various clinical samples like (Table 2)

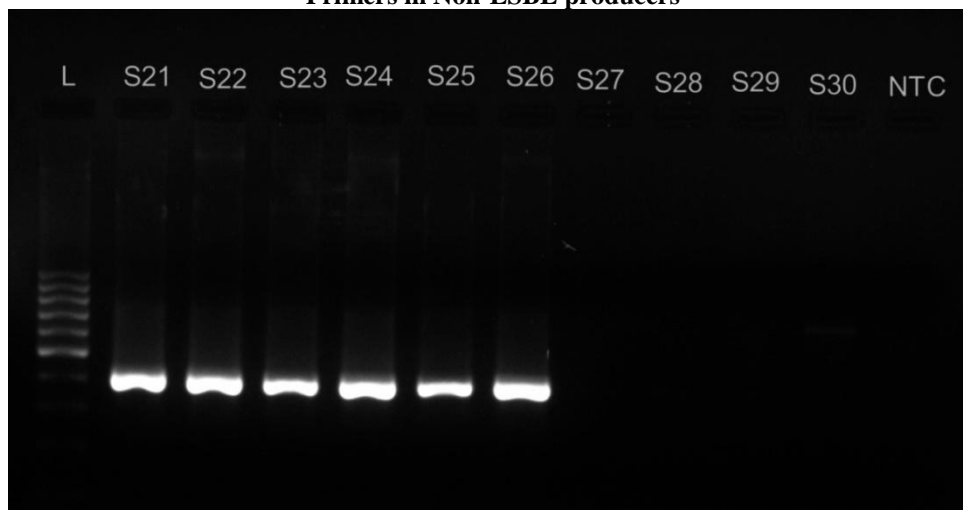
Table 2: Percentage distribution of exotoxin A gene in clinical samples(n=60)

Samples	Total
Pus(42)	30(71.4%)
Urine(11)	7(16.7%)
Sputum(7)	5(11.9%)
Total(60)	42(70%)

Among 203 isolates, 60 were randomly selected (42, 11 and 7 from pus, urine and sputum respectively) which included 30 ESBL and 30 non- ESBL producers for toxA gene expression. Distribution of toxA gene from 60 clinical isolates of *Pseudomonas aeruginosa* showed 70% positivity of which 71.4% were from pus, 16.7% were from urine and 11.9% were from sputum.(Table 2).

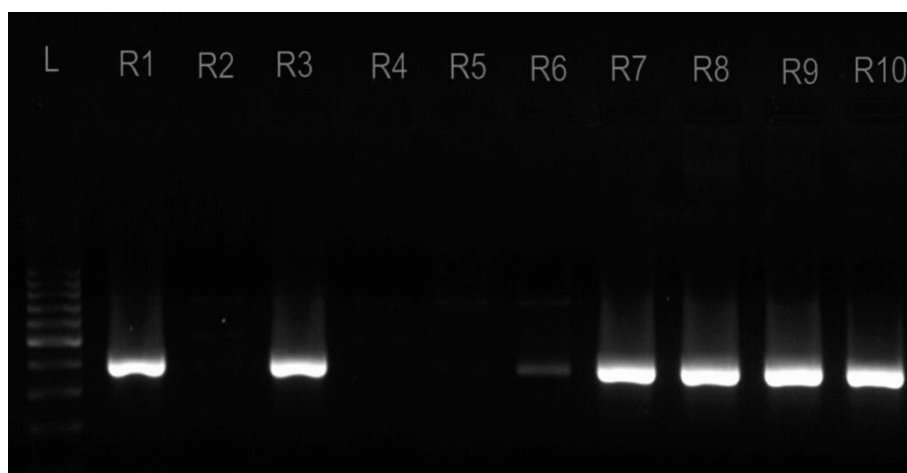
The results of gel electrophoresis have been depicted in figure 1 and 2

Figure 1: Gel Electrophoresis of the Polymerase Chain Reaction Products Using exoA Gene-Specific Primers in Non-ESBL producers



L-Ladder (1 Kb), S21-S26-*toxA* gene positive(396-bp), S27-S30- *toxA* gene negative,NTC-Negative control.

Figure 2: Gel Electrophoresis of the Polymerase Chain Reaction Products Using *exoA* Gene-Specific Primers in ESBL producers



L-Ladder (1 Kb), R1,R3,R6-R10 - *toxA* gene positive(396-bp),R2,R4,R5 – *toxA* gene negative

Table 3: Analysis of exotoxin A gene production among ESBL and Non-ESBL producers

ESBL Production	Exotoxin A gene	
	Positive	Negative
ESBL Positive(30)	23(76.7%)	7(23.3%)
ESBL Negative(30)	19(63.3%)	11(36.7%)
Total(60)	42	18

The p-value is .259796. Not significant as the $p > .05$.

In table 3, analysis of exotoxin A gene production among ESBL and Non-ESBL producers showed, of 30 ESBL positive isolates 76.7% were positive and 23.3% were negative for *toxA* gene production. Among 30 ESBL negative isolates 63.3% were positive and 36.7% were negative for *toxA* gene production. The chi-square statistic is 1.2698. The p-value is .259796. This result is not significant as the $p > .05$.

IV. Discussion

Pseudomonas aeruginosa exhibit large number of virulence factors such as exotoxin A, exoenzyme S, elastase and sialidase. There are cell-to-cell signalling systems which regulates these virulence factors. Exotoxin A inhibits protein biosynthesis and exoenzyme S is secreted by a type III secretion system. The gene *Las B* which encodes zinc metalloprotease has an elastolytic activity on the lung tissue and another gene called *nan1* encoding the enzyme sialidase is responsible for adherence of the organism to the respiratory tract.¹²

In hospital-acquired infections, *Pseudomonas aeruginosa* was reported from 18% to 61% which has been associated with a high morbidity and mortality rate.¹³⁻¹⁵ The pathogenicity and the virulent property of *Pseudomonas aeruginosa* are related to its various adhesion factors and other secreted toxins. Despite using potent antibiotics, invasive nature of *Pseudomonas aeruginosa* infection is often associated with high mortality.¹⁶ In the past decade, acquired multidrug resistance has emerged because of selective antibiotic pressure, and indeed infections caused by multidrug resistant *P. aeruginosa* have been untreatable.¹⁷

Exotoxin A (ETA) is considered one of the most powerful extracellular virulence factors produced by *P. aeruginosa* [18]. Exotoxin A as a potent cytotoxin has got a lethal effect in human by inhibition of protein synthesis, direct cytopathic effects, and interference with cellular immune functions of the host. The *toxA* gene which encodes 68 kDa ETA protein, is an ADP-ribosyltransferase that irreversibly inhibits protein synthesis in eukaryotic cells which results in cell death.^{18,19} The regulatory mechanism of ETA production is an intricate process that involves several regulators and environmental factors.¹⁹

In our study *exoA* gene was found in 70% of isolates similar to another study where they reported 75%.²⁰ Genomic DNA Mini kit system delivers an accurate and fast technique to purify chromosome and plasmid DNA. Khan and Cerniglia²¹ reported that ETA gene was detected in 97% of *Pseudomonas aeruginosa* isolates. The relationship between *toxA* gene and pathogenicity is that it has a direct role in the pathogenesis with tissue damage and decreased phagocytic activity.⁶

In addition to its innate resistance, acquired additional resistance due to plasmids is also a problem in *P. aeruginosa*. Inappropriate and indiscriminate use of antibiotic is associated with plasmid-mediated resistance.²² Similar to Enterobacteriaceae, Pseudomonads also acquire drug resistance by various mechanisms of which, ESBL production is one of them. The usage of antibiotics against this bacteria will be limited because the ESBL producing organisms may carry co-resistance genes for other non- β -lactam antibiotics(rephrase). Thus ESBL enzyme-encoding genes like SHV-2a and TEM-42 is not only found in Enterobacteriaceae family but have been associated with *P. aeruginosa*.^{23,24}

In our study, we compared the exotoxin A expression with that of ESBL and Non-ESBL producers. Of 30 ESBL positive isolates, 76.7% were positive and among 30 ESBL negative isolates 63.3% were positive for *toxA* gene production. The present study showed there was no significant correlation between ESBL producers and exotoxin A gene expression (p-value=0.25).

Few comparative studies were done on exo-toxin A production in clinical and environmental samples, which have proven that higher percentage of exotoxin genes in clinical isolates compared to environmental isolates of *Pseudomonas aeruginosa*.²⁵ Another study on renal pathology by toxin producing parent strain and toxin deficient mutant strain, showed mutant strain to be more virulent to induce renal pathology.²⁶

In our study, there was no significant association between production of *toxA* gene and ESBL production similar to another study conducted by Corehtash et al.²⁰ The simultaneous detection of virulence factor production and antimicrobial resistance mechanism is the contemporary approach for the examination of the microbiological aspects of infections caused by *P. aeruginosa*. Studies have shown that the association between virulence factors and antibiotic resistance may have either positive effect or negative effect or compensatory mutation may occur in due course of time to equilibrate the balance.²⁷ This may have different consequence on the outcome of infections. However in severe infections, extended spectrum beta lactamases (ESBLs) production and expression of some virulence factors may work in harmony, resulting in failure in treatment. Therefore, the regular detection of ESBLs by conventional methods should be carried out in every laboratory.

V. Conclusion

To conclude, more than 70% clinical isolates of *Pseudomonas aeruginosa* were positive for *toxA* gene. However the association between exotoxin A gene expression and resistance mechanism like ESBL production was not statistically significant in our study, which may indicate that there need not be always a direct correlation between virulence gene and antibiotic resistance and they may act independently. The fact that there appears to be no direct correlation between virulence factors is not surprising incidence as any combination of virulence factors aids infection and horizontal transmission or exchange of genes for resistance mechanism.

Acknowledgements

The authors would like to thank Dr. Adithan Chandrasekaran, Director, Centre for Interdisciplinary Research Facility (CIDRF) and Ms. B. Deivanayagi, Lab technician, CIDRF of Sree Balaji Vidyapeeth University for their support in carrying out the molecular work up.

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Pramodhini S "Comparative analytic study of exotoxin A gene production among ESBL and non-ESBL producing clinical isolates of Pseudomonas aeruginosa.". "IOSR Journal of Dental and Medical Sciences (IOSR-JDMS), Volume 17, Issue 2 (2018), PP 41-46.