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Original Article

Microbiology Section

Prevalence of *exoT* Gene in *Pseudomonas aeruginosa* Isolated from Various Clinical Samples: A Cross-sectional Study

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ABSTRACT

Introduction: *Pseudomonas aeruginosa (P. aeruginosa)* is one of the most frequently co-infecting bacteria reported. Development of drug resistance, biofilm formation, cell associated factors make the *P. aeruginosa* more virulent. Type III secretion system controls expression of genes. *P. aeruginosa* chromosome harbours *exoT, exoS, exoU*, and *exoY* virulent genes. Gene *exoT* plays an important role in causation of infection. The identification of virulent markers of pathogens for identification of acute and chronic infections at early stage remains a critical area and still need large research.

Aim: To study the prevalence of *exoT* gene encoding exotoxin T in *P. aeruginosa* from clinical samples.

Materials and Methods: A descriptive cross-sectional research was conducted during January 2015 to March 2016 at the Department of Microbiology in Dr. D. Y. Patil Medical College, Pimpri, Pune, Maharashtra, India. Various clinical samples were processed using standard laboratory methods. The statistical analysis was done by using Chi-square test. Strains of *P. aeruginosa* isolated from various clinical samples were identified using standard laboratory methods, and *exoT* gene was detected by Polymerase Chain Reaction (PCR) and gel electrophoresis technique.

Results: Out of 30 strains of *P. aeruginosa*, 20 (66.67%) were isolated from male and 10 (33.33%) from female patients. Most of them belonged to the age group 41-60 years (46.67%). The *exoT* gene occurred in 20 (66.67%) isolates of *P. aeruginosa*, while 10 (33.33%) showed negative amplification results. Out of 20 *exoT* genes in *P. aeruginosa*, 17/20 (85%) were detected from male and 3/10 (15%) from female patients.

Conclusion: Gene *exoT* of *P. aeruginosa* plays the crucial role in causation of disease. It is concluded that *exoT* gene can be a notable virulent element expressed by 66.67% of *P. aeruginosa* clinical isolates. The proven role of *exoT* virulence gene in the pathogenicity of *P. aeruginosa* would help in understanding the prognosis of *Pseudomonas* infection and designing an effective treatment and vaccine against the *Pseudomonas* infections to prevent them.

Keywords: Exotoxin T, Gram negative bacteria, Polymerase chain reaction

INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is an actively motile, biofilm forming gram negative human opportunistic pathogen. It exhibits multidrug resistance and is widely connected with hospital acquired infections [1]. They are resistant to multiple antibiotics due to acquired or inherent determinants. It can cause acute and chronic infections [2]. *P. aeruginosa* causes serious infections such as endocarditis, pneumonia, septicaemia, wound infections, cystitis, blood stream infections, urinary tract infection and surgical site infections [3,4]. Biofilm formation, development of drug resistance, various secreted toxins, proteases, pyocyanin, exotoxins and different cell associated factors make *P. aeruginosa* more virulent [5]. *P. aeruginosa* has the ability to colonise the respiratory tract and is one of the members of normal flora of nasopharynx [6]. *P. aeruginosa* infections are more frequently observed in cystic fibrosis and in weakened immune patients. In a recent study, researcher documented *P. aeruginosa* as a common co-infecting pathogen in patients [7].

P. aeruginosa continues to exist in different environmental states because of different virulence factors and metabolic properties [8]. Toxins are released by passive transport from the cells secreted by one of the three secretion systems namely, Type I Secretion System (T1SS), Type II Secretion System (T2SS) and Type III Secretion System (T3SS). Type III secretary system plays a key role in determining virulence [9]. The gram negative bacteria have a complex T3SS which is an essential machinery of *P. aeruginosa* to inject exotoxin T (*exoT*), exotoxin S (*exoS*) and exotoxin U (*exoU*) virulence factors directly into host cells and can evoke different responses from the host suitable for spreading of infection [10,11]. Exotoxin produced during release and escape of pathogen mainly attacks host kinases and is responsible for adhesion, phagocytosis, with spreading type of infection from lung to the liver in experimental animal [9,12].

Very few researchers in India have focussed on *exoT* gene encoding exotoxin T, virulence factor of T3SS of *P. aeruginosa*. In previous similar study in India, the researcher documented 84% prevalence of *exoT* gene in *P. aeruginosa* strains obtained from various clinical samples [13]. Keeping these facts in mind, this study was designed to study the prevalence of *exoT* gene in *P. aeruginosa* obtained from different clinical samples in a tertiary care hospital.

MATERIALS AND METHODS

Present descriptive cross-sectional research was conducted during January 2015 to March 2016 at the Department of Microbiology in Dr. D. Y. Patil Medical College, Hospital and Research Centre, Pimpri, Pune, Maharashtra, India. Different clinical samples (pus, urine, sputum, blood and body fluids) were received from various wards irrespective of age and gender for routine culture and sensitivity tests. The isolates were processed and confirmed *P. aeruginosa* strains were screened for detection of *exoT* gene by PCR and gel electrophoresis techniques. The study was done after approval from Institutional Ethical Committee of Dr. D. Y. Patil Medical College, Hospital and Research Centre, Pimpri, Pune, Maharashtra, India.

Inclusion criteria: Samples showing *P. aeruginosa* as single causative agent of infection were included in this study. Total 30 strains were included in this research study.

Exclusion criteria: Samples showing mixed growth were excluded from this study.

Study Procedure

Isolation and identification of *P. aeruginosa*: All clinical samples were inoculated onto MacConkey agar, Nutrient agar and Blood agar plates. Inoculated plates were incubated at 37ºC for 24 hours. After obtaining the growth, *P. aeruginosa* was identified by studying colony characteristics, production of pyocyanin pigments, grape like odour, growth at 42ºC, motility test, gram staining, and positive oxidase, citrate, and catalase tests [14].

Extraction of DNA: For the detection of *exoT* gene, chromosomal Deoxyribonucleic Acid (DNA) from the 30 clinical strains of *P. aeruginosa* clinical isolates was extracted and DNA purification was carried out using a commercial available DNA extraction kit (Ganeaid-Presto™ Mini gDNA bacteria Kit) as indicated by manufacturer's instructions.

Polymerase Chain Reaction (PCR): The sequences of the primers used in PCR for detection of *exoT* gene and its molecular weight are shown in [Table/Fig-1] [15,16].

Gene	Primer sequence	Amplicon size	Length (bp)
exoT	Forward 5'-AATCGCCGTCCAACTGCATGCG-3'	22	152
	Reverse 5'-TGTTCGCCGAGGTACTGCTC-3'	20	

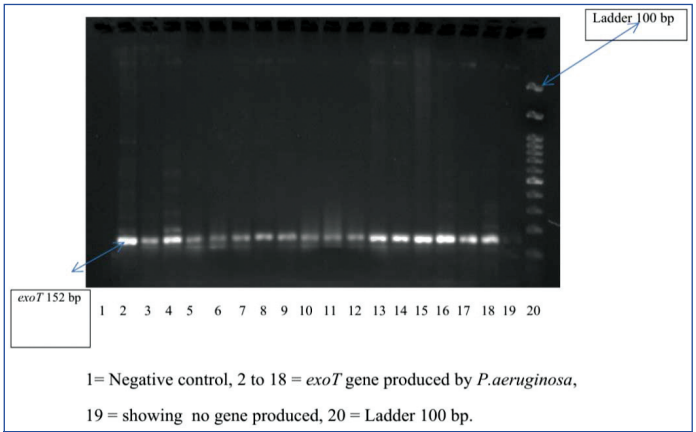
[Table/Fig-1]: The Primer sequence used for the detection of exoT genes.

For all PCRs, the DNA extracted from *P. aeruginosa* under study was used as templates. PCRs were carried out in 25 µL mixture containing 12.5 µL mastermix (Geneaid-Presto™Mini gDNA bacteria Kit), 1.5 µL forward primers, 1.5 µL reverse primers, 2.0 µL DNA template and 7.5 µL distilled water [Table/Fig-2].

Gene	Initial denaturation	No. of cycles	Denaturation in each cycle	Annealing	Primer extention	Final extention
exoT	95ºC, 2 min	36	95ºC, 30 sec	58ºC, 30 sec	72ºC, 30 sec	72ºC, 5 min

[Table/Fig-2]: Polymerase Chain Reaction (PCR) conditions.

Gel electrophoresis: Electrophoresis was done using PCR products of *P. aeruginosa*. To prepare agarose gel, 2% agarose with ethidium bromide was used, as it shows clear resolution for small fragments. The quantity of agarose used for a gel preparation was around 250 mL. Images of PCR products were detected using transillumator by UV illumination is shown [Table/Fig-3]. PCR products were estimated by comparing with the 100 bp DNA molecular size markers [17].



[Table/Fig-3]: Showing gel electrophoresis and amplification products of virulence gene (*exoT*) of *Pseudomonas aeruginosa* clinical isolates.

STATISTICAL ANALYSIS

The statistical analysis was done by using Chi-square test online by graphpad prism 9.2.0.332. The calculated p-value was 0.0093 which is significant.

RESULTS

Out of 30 strains of *P. aeruginosa*, 20 (66.67%) were isolated from male and 10 (33.33%) from female patients. Most of the subjects belonged to 41-60 years of age group (46.67%). Out of 20 *exoT* genes in *P. aeruginosa*, 17/20 (85%) were detected from male and 3/10 (15%) from female patients [Table/Fig-4].

Age group (years)	Male	Female
0-20	00	00
21-40	02	01
41-60	12	02
61-80	03	00
Total	17	03

[Table/Fig-4]: Age and gender wise distribution of prevalence of *exoT* gene in *P. aeruginosa* (N=20).
Chi square 6.769, df-1, p-value=0.0093, p-value is statistically significant

The prevalence of *exoT* gene was 66.67%. Out of 30 strains of *P. aeruginosa* clinical isolates, 20 (66.67%) showed amplification of *exoT* gene and 10 (33.33%) strains showed negative amplification [Table/Fig-5].

S. No.	Sample	Case	*exoT*	S. No.	Sample	Case	*exoT*
1	Pus	Maxilla	+	16	Urine	UTI	+
2	Pus	CSOM	+	17	Pus	CSOM	+
3	Pus	NHTU	+	18	Pus	CSOM	-
4	Pus	Hydrocele	+	19	Urine	UTI	-
5	Urine	CUTI	+	20	Blood	Fever	-
6	Urine	UTI	+	21	Pus	Leg abscesses	+
7	Pus	Leg cellulitis	+	22	Pus	NF	-
8	Pus	DFU	+	23	Pus	NF	-
9	Fluid	COPD	+	24	Sputum	RTI	+
10	Pus	NF	+	25	Pus	TA	-
11	Pus	DFU	+	26	Pus	DFU	-
12	Pus	CSOM	+	27	Pus	DFU	-
13	Urine	UTI	+	28	Sputum	RTI	+
14	Sputum	RTI	+	29	Blood	Pneumonia	-
15	Sputum	RTI	+	30	Pus	DFU	-

[Table/Fig-5]: Distribution of *exoT* genes of 30 *Pseudomonas aeruginosa* clinical Isolates in respect to sites of Infections.
CSOM: Chronic suppurative otitis media; NHTU: Non healing tropic ulcer; UTI: Urinary tract infection; NF: Necrotizing fascitis; COPD: Chronic obstructive pulmonary disease; DFU: Diabetes foot ulcer; RTI: Respiratory tract infection; TA: Traumatic amputation; CAUTI: Catheter associated UTI

Out of 30 strains of *P. aeruginosa* 11/18 (36.67%) isolates from pus detected *exoT* gene followed by urine 4 (80%) out of five, sputum 4/4 (100%), body fluid 1/1 (100%) and blood 0/2 (0%) [Table/Fig-6].

S. No.	Source	*exoT* (%)
1	Pus	11 (61.11)
2	Urine	4 (80)
3	Sputum	4 (100)
4	Blood	0 (0)
5	Body fluids	1 (100)
6	Total	20 (66.67)

[Table/Fig-6]: Showing distribution of *exoT* genes *Pseudomonas aeruginosa* Clinical Isolates (Pus n=18, Urine n=5, Sputum n=4, Blood n=2, Fluid n=1) in different samples, Total n=30).

DISCUSSION

P. aeruginosa is one of the major and well identified nosocomial pathogen. It has capability to survive and multiply with minimal nutrients. It can cause severe infection in hospitalised patients. *P. aeruginosa* is one of the most common co-infecting pathogen and exotoxin T is virulence factor that make *P. aeruginosa* more virulent for host cells. This descriptive research study was undertaken to detect *exoT* gene encoding exotoxin T in *P. aeruginosa* clinical isolates. T3SS is a basic and important weapon of *P. aeruginosa* and several other gram negative organisms for survival and causation of

disease. The T3SS is a needle like sharp tiny machine that carry and deliver effector proteins (exotoxins) directly into targeted cells of the host to start disease. These delivered exotoxin precipitate and continue progress of infection by changing normal functions of target cells, such as constant movement of network of protein filaments and microtubules in the cytoplasm, reactions of cells to inflammatory stimuli, signalling pathways and secretory trafficking [18]. T3SS complex cellular nano machine is a key weapon of several pathogenic gram negative bacteria which works in a systematic planned mode of action and can alter the target cell in number in irregular manner. T3SS has gradually developed because of the pressure of the survival within infected cells.

Virulence factors injected by T3SS into host cells play important role for *P. aeruginosa* to be more virulent [19,20]. The gene *exoT*, modify the actin cytoskeleton, inhibit migration and multiplication of cell. Exotoxin T prevents adhesion, phagocytosis, excessive multiplication and stop epithelial barrier that help *P. aeruginosa* to cause spread type of infections [21]. Gene *exoT* is an important virulence gene encoding exoenzyme T. Therefore, detection of *exoT* gene is important while determining pathogenicity of *P. aeruginosa* in different type of infections.

In this research study, authors aimed to determine the prevalence of *exoT* virulent gene in 30 strains of *P. aeruginosa* isolated from various clinical samples in a tertiary care hospital. PCR and gel electrophoresis technique was used for the detection of *exoT* gene in *P. aeruginosa* under study. In this study, the prevalence of *exoT* gene was 66.67%. Out of 30 strains of *P. aeruginosa* clinical isolates from the different clinical conditions, 20 (66.67%) detected *exoT* gene in all spreading type of infections caused by *P. aeruginosa. P. aeruginosa* isolates from pus (61.11%), sputum (100%), urine (80%) and bodyfluids (100%) samples showed presence *exoT* gene. Out of 30, 10 (33.33%) strains were found to be negative for *exoT* gene.

In other similar study in southern India, the prevalence of *exoT* was recorded as 84% [13]. Many researches all over the world studied the prevalence of *exoT* genes as an epidemiological marker in pathogenic *P. aeruginosa* causing different type of infections. The prevalence of *exoT* genes has been found to be variable in *P. aeruginosa* isolates obtained from different infections in the world. Prevalence of *exoT* gene recorded in Iran was 36.27% [22] and in Egypt and Romania, prevalence of *exoT* in *P. aeruginosa* clinical isolates were recorded as 100% [12,23].

Role of *exoT* gene is crucial in the causation of spreading type infections. T3SS virulence factors are responsible for seriousness of the infections with raised death rate [24]. The proven role of *exoT* virulence genes in the pathogenicity of *P. aeruginosa* would help in understanding the prognosis of *Pseudomonas* infections and designing an effective vaccine against the pseudomonas infections to prevent them. This may help in epidemiological study, deciding the treatment course for the infections caused by *P. aeruginosa*. These findings may help in identifying virulent gene targets for immune intervention which could regulate the severity of the host response and its effect on impairment caused by *P. aeruginosa*.

The identification of virulent markers of pathogens for identification of acute and chronic infections at early stage remains a critical area and still need large research. Such type of research studies and findings facilitate the prevention of infections caused by bacteria and can be very useful to control the *Pseudomonas* infections.

Limitation(s)

All the *P. aeruginosa* strains under study were obtained from Inpatient Department (IPD) only. Therefore, prevalence of *exoT* gene in *P. aeruginosa* could not be studied in Outpatient Department (OPD) patient. Larger sample size of both IPD and OPD patients would have provided better status of prevalence of *exoT* virulent gene marker as well as difference and significance.

CONCLUSION(S)

Gene *exoT* encoding ExotoxinT plays a very crucial role in the causation of disease. The proven role of *exoT* virulence genes in the pathogenicity of *P. aeruginosa* would help in understanding the prognosis of *Pseudomonas* infections and designing an effective vaccine against the *Pseudomonas* infections to prevent them. This may help in epidemiological study, deciding the treatment course for the infections caused by *P. aeruginosa*. In future trends in diagnostic microbiology, focus should be on development of rapid tests required for detection of virulence factors which are important epidemiological markers apart from identification and antimicrobial susceptibility tests.

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