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TO STUDY THE MOLECULAR CHARACTERIZATION OF *EXOT* GENE IN *PSEUDOMONAS AERUGINOSA* AND ITS ANTIBIOGRAM WITH THE ABILITY OF BIOFILM FORMATION

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ABSTRACT

Introduction: The emergence of *Pseudomonas aeruginosa* strains that are resistant to drugs has made medical treatment more difficult. The development of biofilms and its antimicrobial resistance are two virulence factors that support the persistence of chronic infections. Clinical isolates of P. *aeruginosa* have been shown to express the gene ExoT, which is a significant virulent element that contribute to the causation of infection.

Aim and Objective: To Study the Molecular characterization of ExoT Gene from *Pseudomonas aeruginosa* and its Antibiogram with the ability of Biofilm Formation in patients.

Material & Methods: This was a cross sectional study conducted in the Department of Microbiology at a tertiary care centre for a period of 12 months 1.e, April 2023 to April 2024. A total of 130 *Pseudomonas aeruginosa* isolated from different clinical samples including urine, sputum, ear swab, wound swab , pus were identified by standard microbiological techniques. The isolates were further tested for MBL by Imipenem – EDTA combined disc test and MBL E-test (Imipenem). The DNA was isolated by Qiagen DNA extraction kit as per the manufacture's guidelines and the resistant gene Exo T was detected by the conventional PCR.

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Results: A total of 400 clinical samples were studied out of which 130 *P. aeruginosa* isolates were obtained. The prevalence rate of *Pseudomonas aeruginosa* was observed to be 32.5%. It was observed that the Sensitivities of Colistin was (96.6%), Piperacillin-tazobactam (77.6%), Amikacin (77.6 %), and cefepime (74.6 %) were found to be the most effective Antibiotics. The resistance to ciprofloxacin was (46.1%), Levofloxacin (53.8%), Gentamicin(62.3%), Imipenem (67.6%), Tobramycin(68.4%), Ceftazidime (68.4%). There were 25(19.2%) were MBL positive by Imipenem – EDTA combined disc test, and 24(18.4%) by MBL E- test. In the present study it was observed that 67 (51.5%) of the *Pseudomonas aeruginosa* isolates showed biofilm formation. The molecular characterization confirms 52 (40%) expression exoT gene.

Conclusion: In our investigation, the MBL E-Test was determined to be the most effective phenotypic technique for detecting MBL. The established significance of ExoT virulence genes in the pathogenicity of *P. aeruginosa* would aid in the treatment and prognosis of Pseudomonas infections.

Keywords: Molecular Characterization, Qiagen Kit, DNA, PCR, Exo T, MBL E-Test

INTRODUCTION

Pseudomonas aeruginosa is a major source of human illness, particularly in those with weaker immune systems [1]. This bacteria forms biofilms on biotic or abiotic surfaces, requires little feeding, and can tolerate a wide range of environmental conditions [2, 3]. Pseudomonas colonises catheters, skin wounds, ventilator-associated pneumonia, and respiratory infections in patients with cystic fibrosis (CF) in human hospitals, making it a leading source of nosocomial infections [4]. Pseudomonas organisms colonise an area when the fibronectin layer that surrounds the host cells is damaged by injury or infection. They are resistant to multiple antibiotics due to acquired or inherent determinants. It can cause acute and chronic infections [2].

The rising prevalence of multidrug-resistant (MDR) strains of *P. aeruginosa* has complicated medical management, which is a global issue. *P. aeruginosa* produces biofilms in the airways if it is not eliminated during the initial infection phase [5]. One of the main factors contributing to chronic infections is the formation of biofilm, which are organised bacterial communities

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embedded in an extracellular polymeric matrix adhered to surfaces [6,7]. Biofilms offer bacteria a protective lifestyle and are very difficult and expensive to treat with antibiotics .The biofilm components of *P. aeruginosa* are composed of at least three distinct exopolysaccharides, including alginate, Psl and Pel [8].

Biofilm formation, development of drug resistance, various secreted toxins, proteases, pyocyanin, exotoxins and different cell associated factors make P. aeruginosa more virulent [5].

The virulence factors can be chemical or proteinaceous, and either cell-associated or secreted. Proteinaceous virulence factors are often secreted through one of the five protein secretion systems so far described as *P. aeruginosa*: type I, II, III, V [9] and the recently discovered type VI [10]. Especially the type III secretion system (TTSS), which injects effector proteins directly into the eukaryotic host cell cytoplasm, has been associated with high virulence. Infection with a type III secreting isolate has been shown to correlate with severe disease [11], and type III secretion (TTS) in lower respiratory and systemic infections is associated with an increased mortality rate

MDR, XDR, and PDR variations exhibit significant levels of intrinsic resistance to antimicrobial medicines via efflux pumps, biofilm development, aminoglycoside modifying enzymes, and, in certain cases, chromosomal gene mutation (ESBL and AmpC hyper expression). Pseudomonas spp. can also develop resistance through the horizontal gene transfer pathway, which is responsible for class B carbapenamase (MBL) [12].

Genes responsible for drug resistance are located on integrons which is frequently located in plasmids or transposons and these genes can shift very often and contributes to the dissemination of resistance mechanism around the world [13].

P. aeruginosa also has a large number of other virulence factors such as exotoxin A (*exoA*), alkaline protease (*aprA*), exoenzyme S, U, and T (*exoS*, *exoU*, *exoT*), elastase and sialidase, which are *exoA* gene and virulence factor *exoS* secretions by a type III section system [14,15].

One of the reasons for the poor clinical outcomes of *P. aeruginosa* infections is thought to be virulence factors, especially the Type III secretion system (T3SS) which is considered an important contributor to cytotoxicity and the invasion process. This system allows these bacteria to directly inject effector proteins into eukaryotic cells. At present, four effector proteins have been identified:

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ExoU, a phospholipase; ExoY, an adenylate cyclase; and ExoS and ExoT, which are bifunctional proteins. ExoT and ExoY are encoded by almost all strains, therefore might be considered an inevitable component of *P. aeruginosa* virulence [14].

ExoT is a bi-functional protein possessing an N-terminal GTPase-activating protein (GAP) domain, which inhibits RhoA, Rac1, and Cdc42, small GTPases, and a C-terminal ADP-ribosyltransferase (ADPRT) domain, which targets Crk adaptor proteins and PGK1 glycolytic enzyme [16]. Due to its multiple targets, ExoT performs a number of distinct virulence functions for *P. aeruginosa* [17-20]

Drug-resistant phenotypes have emerged as a result of Pseudomonas spp.'s ability to produce a diverse spectrum of drug resistance mechanisms. This provides a challenge for our clinician

while treating a major infection. This type of scenario results in the development of phenotypes that produce diverse pharmaceutical resistance mechanisms in order to prevent treatment failure and hospital-acquired infections [12].

Therefore the present study was undertaken to study the molecular characterization of Exo T gene from *Pseudomonas aeruginosa* and its antibiogram with the ability of biofilm formation of patients attending a tertiary care centre.

MATERIAL AND METHODS

This was a Cross sectional study carried out in the Department of Microbiology, for a period of 1 year i.e, between April 2023 to April 2024. A Total of 400 clinical isolates from different clinical samples including urine, sputum, ear swab, wound swab , pus were identified by Standard microbiological techniques and biochemical methods, including pigment production in agar, oxidase and catalase tests, reactions in triple sugar iron (TSI) agar, SIM (sulfide, indole, motility), and oxidative-fermentative (OF) media (Merck, Darmstadt, Germany), and finally, growth at 42 °C [21]. Samples were processed as soon as received in laboratory. In cases where a delay was expected, the sample was refrigerated for up to 4 hours at 4°C.

All clinical samples were inoculated onto MacConkey agar, Nutrient agar and Blood agar plates. Inoculated plates was incubated at 37^{o}C for 24 hours. After obtaining the growth, *P.aeruginosa* was identified by studying colony characteristics, production of pyocyanin pigments, grape like odour, growth at 42^{o}C, motility test, Gram staining, and positive oxidase, citrate, and catalase tests

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was performed according to the latest CLSI guidelines . And the isolates were further tested for MBL by Imipenem – EDTA combined disc test and MBL E-test (Imipenem) according to the CLSI guidelines (CLSI, 2022) [22].

INCLUSION CRITERIA:-

1. This study included all patients from both IPD and OPD who had a positive Pseudomonas culture.
2. Patients of all ages and genders participated in our study.
3. This investigation comprised samples in which P. aeruginosa was the sole cause of infection.

EXCLUSION CRITERIA:-

Samples showing mixed growth was excluded from this study.

The patients demographic profile and consent was taken, and the Ethical clearance was duly obtained from the Institutional Ethical Committee.

Fig No. 1 : *Pseudomonas aeruginosa* ATCC Strain (27853) Used for Quality Control, which was Subculture on Nutrient Agar Plate, MacConkey Agar, Cled Agar and Blood Agar

The Susceptibility of isolates to different antibiotics was determined by disk diffusion agar method on cation-adjusted Mueller–Hinton agar (Merck, Darmstadt, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) recommendations 2023 [22]. Antibiotic disks (MAST Diagnostics, Merseyside, UK) tested were ceftazidime (CAZ, 30 μg), piperacillin/tazobactam (PTZ, 100 μg/10 μg), ciprofloxacin (CIP, 5 μg), levofloxacin (LEV, 5 μg), gent amicin (GM, 10 μg), amikacin (AK, 30 μg), tobramycin (TOB, 10 μg), imipenem (IMI, 10 μg), and meropenem

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(MEM, 10 μg).

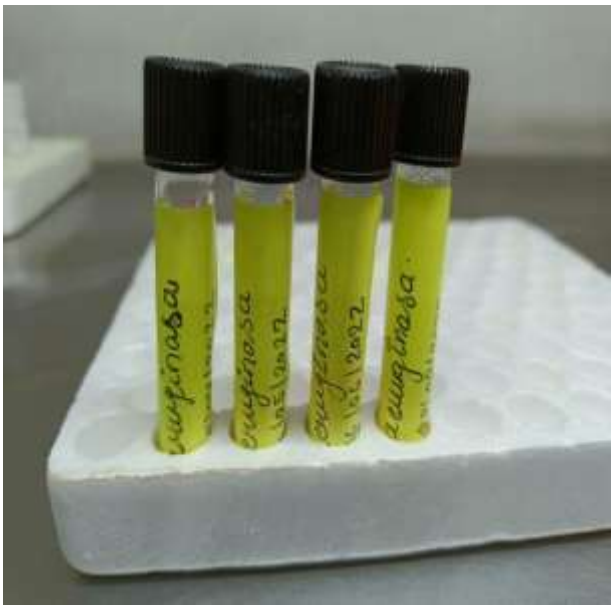
Escherichia coli ATCC 25922 was used as a control for susceptibility testing. Multidrug-resistant *P. aeruginosa* (MDR-PA) was defined as resistant to more than one antimicrobial agent in three or more antimicrobial categories [23-25].

The detection of Biofilm formation in *Pseudomonas aeruginosa:*

The Biofilm Formation Steps:

1. A loopful of test organism was added to 10 ml of Trypticase Soy Broth with 1% Glucose in the test tubes.
2. Tubes were incubated at 37°C for 24 hours.
3. Following incubation, tubes were decanted, washed with phosphate buffer (normal saline), and dried.
4. The tubes were stained with 0.1% crystal violet.
5. Remove excess discoloration with distilled water.
6. Tubes were dried in an inverted orientation.
7. Scoring for tube method was based on control strain results.

• Positive biofilm production occurs when a visible thick film forms between the tube's wall and bottom.



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- **Fig No. 2 : The purified isolated colony of *Pseudomonas aeruginosa* was stabbed in the BHI agar with 10 % glycerol and stored at -4º c for further Analysis of *Pseudomonas aeruginosa***

GENOTYPIC METHOD:

The Molecular Detection of DNA extraction was done to detect the presence of exo T gene in *Pseudomonas aeruginosa* from the clinical isolates.

Molecular Identification of *Exo T* gene

The DNA was isolated using the Qiagen DNA Extraction Kit as per the manufactures guidelines. The DNA was eluted in 60 µl elution buffer and preserve at -20 °C till PCR analysis. For amplification of the target gene, PCR was carried out in a 50 µL reaction mixture. The primers were purchased from "**Saha gene"** and was reconstituted with sterile double distilled water based on the manufacturer's instruction.

The kit works on the principle of selective binding of DNA/RNA to silica membrane in a spin column micro-centrifuge tube. DNA/RNA is rapidly purified by centrifugation procedure. Briefly, the specimen is lysed in the lysis bufler, then the released nucleic acid is adsorbed to a silica membrane in a spin subsequently released using the elution buffer.

Kit components:

S.No	Component	Pack size 50 tests
1	Lysis Buffer	10ml
2	Lysis Enhancer	1ml
3	Binding Buffer	15ml
4	Wash Buffer	60ml
5	Elution Buffer	5ml
6	Spin Column	2X25 Nos
7	IFU	1 No.

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Figure No. 3: The Exo T primer from the Saha gene

Polymerase Chain Reaction (PCR): After the DNA Extraction, the PCR was done for the gene detection. The sequences of the primers used in PCR for detection of exoT gene and its molecular weight are mentioned in the Table no. 1.

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

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	Reverse 5'- TGTTCGCCGAGGTACTGCTC-3'	20	

Table no. 1: The Primer sequence used for the detection of *ExoT* genes

ATCC 27853 was used as Positive Control (PC), while nuclease free water will be used as Negative control (NC).

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 no. 2 : The Polymerase Chain Reaction (PCR) conditions.

The above was the cycling conditions used for the PCR.

Gel electrophoresis : The Agarose Gel Electrophoresis was performed in order to identify the Purified PCR Product which was previously identified by its amplified DNA fragments. The resulting PCR product was subjected to 1 % agarose gel electrophoresis and visualized by Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories Inc., Hercules, CA, USA). A 1 kb DNA Ladder (Thermo Fisher Scientific ™, Waltham, MA, USA) was used as the marker to evaluate the PCR product of the sample [26].

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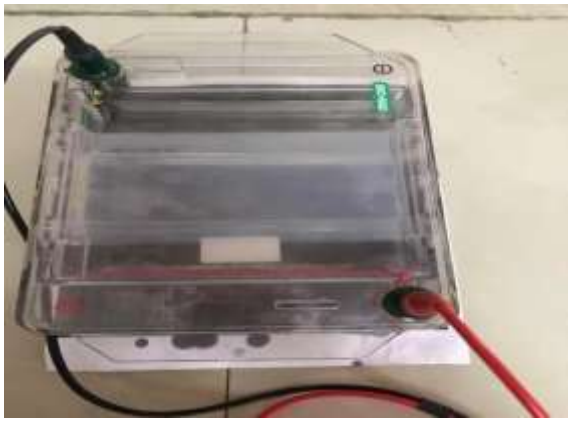

Fig No.4: Gel Electrophoresis for the DNA Extraction

Statistical analysis

The statistical analysis was done by *t*-test using SPSS 20 software in order to find the independence of the variables or whether they had similarities in their MIC values with $P < 0.005$.

RESULTS

In the present study a total of 400 clinically suspected cases from both IPD & OPD were included out of which 130 were culture positive for *Pseudomonas* infection. [Table No. 3]. Therefore, the prevalence rate of *Pseudomonas aeruginosa* was found to be 32.5%.

S.No.	Type of Isolates	Total No. of samples (n=400)	Percentage
1.	*Pseudomonas aeruginosa*	130	32.5%
2.	Other Isolates	270	67.5%

Table No. 3: Total Number of Cases

From the present study it was observed that the Males 84 (64.6%) were more affected with the infection as compared to the Females 46 (35.3%). It was also noted that the age group of 21-30

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years of age followed by 31-40 was affected the most. In the age group of 0-10 years and above 71 years was the least affected with the *Pseudomonas aeruginosa* infection.

S.NO.	GENDER	TOTAL NO. OF ISOLATES (N=130)	PERCENTAGE
1.	Male	84	64.6%
2.	Female	46	35.3%

Table No. 4: Genderwise distribution of the *Pseudomonas aeruginosa* Isolates

S.NO.	Age	No. of Isolates (n=130)	Percentage
1.	0-10	5	3.8%
2.	11-20	17	13%
3.	**21-30**	**48**	**36.9%**
4.	**31-40**	**33**	**25.3%**
5.	41-50	10	7.6%
6.	51-60	8	6.1%
7.	61-70	6	4.6%
8.	≤ 71	3	2.3%

Table No. 5: Agewise distribution of the *Pseudomonas aeruginosa* Isolates

It was also noted that the age group of 21-30 years of age followed by 31-40 was affected the most. In the age group of 0-10 years and above 71 years was the least affected with the *Pseudomonas aeruginosa* infection.

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It was also observed that the maximum number of isolates observed were from the Urine sample (49.2%) followed by the pus (24.6%) and sputum (14.6%), Et- secreation (6.1%) and throat swab (3.8%), and least was from the blood (1.5%).

S.No.	Type of Isolates	Number of Isolates (N=130)	Percentage
1.	Pus	32	24.6%
2.	Urine	64	49.2%
3.	Sputum	19	14.6%
4.	Throat Swab	5	3.8%
5.	Et-Secretion	8	6.1%
6.	Blood	2	1.5 %

Table No. 6: Type of Clinical Isolates Detected from *P.aeruginosa*

Antibiotic susceptibility testing was performed by Kirby bauer Disk diffusion method as per the CLSI guidelines 2023. The antibiotic discs was placed on the Muller–Hinton agar which were previously inoculated with test strains and incubated at 37 °C for 16–18 h. After incubation, inhibition zones was recorded as the diameter of the clear zones around the disc and interpreted according to performance standard for antimicrobial disk susceptibility test as per the CLSI guidelines.

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Fig No. : 5 : The Antimicrobial Disk Susceptibility Test

It was observed that the Sensitivities of Colistin was (96.6%), Piperacillin-tazobactam (77.6%), Amikacin (77.6 %), and cefepime (74.6 %) were found to be the most effective Antibiotics. The resistance to ciprofloxacin was (46.1%), Levofloxacin (53.8%), Gentamicin(62.3%), Imipenem (67.6%), Tobramycin(68.4%), Ceftazidime (68.4%).

The AST was performed, and it was discovered that colistin and polymixin-B were the most sensitive medicines. Amikacin is the most sensitive aminoglycoside against *P. aeruginosa.* Amikacin was meant to be a poor substrate for enzymes that cause inactivation by phosphorylation, adenylation, or acetylation, but certain organisms have evolved enzymes that also inactivate this drug. Amikacin appears to be a viable treatment for Pseudomonal infections. As a result, its usage should be limited to severe nosocomial infections in order to prevent the rapid establishment of resistance strains.

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The detection of Biofilm formation in *Pseudomonas aeruginosa*

In the present study it was observed that 67 (51.5%) of the *Pseudomonas aeruginosa* isolates showed biofilm formation.

Isolates	**Biofilm Producer**	**Percentage**	**Non Biofilm Producer**	**Percentage**
Pseudomonas aeruginosa	67	51.5%	63	48.4%

Table no. 7: The Biofilm producer in *Pseudomonas aeruginosa*

There were 25(19.2%) were MBL positive by Imipenem – EDTA combined disc test, and 24(18.4%) by MBL E- test.

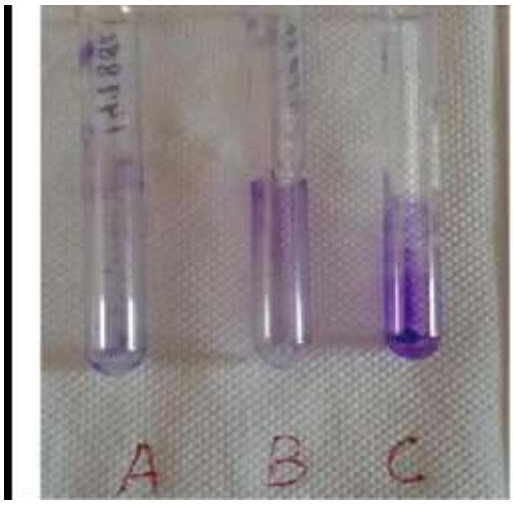

Figure 6: The tube Method for the Biofilm formation in *pseudomonas aeruginosa*

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A: Sample Negative for the Biofilm Formation

B: Sample Positive for the Biofilm

C: Positive Control for the Biofilm formation

The reference strain *P. aeruginosa* (PA01) was also used as a positive control of the test because this strain has been characterized as a biofilm producer.

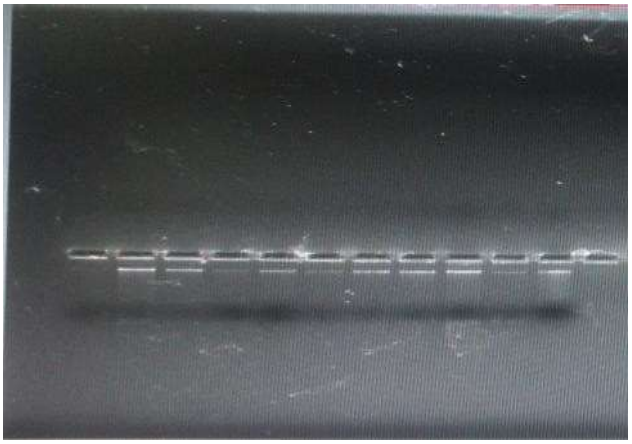

Fig:No. 7 The DNA Extraction in Agarose gel

In the present study it was observed that out of 130 Pseudomonas isolates there were 52 (40%) which expressed ExoT gene.

L (Ladder) , L1,L2,L3,L4, L5

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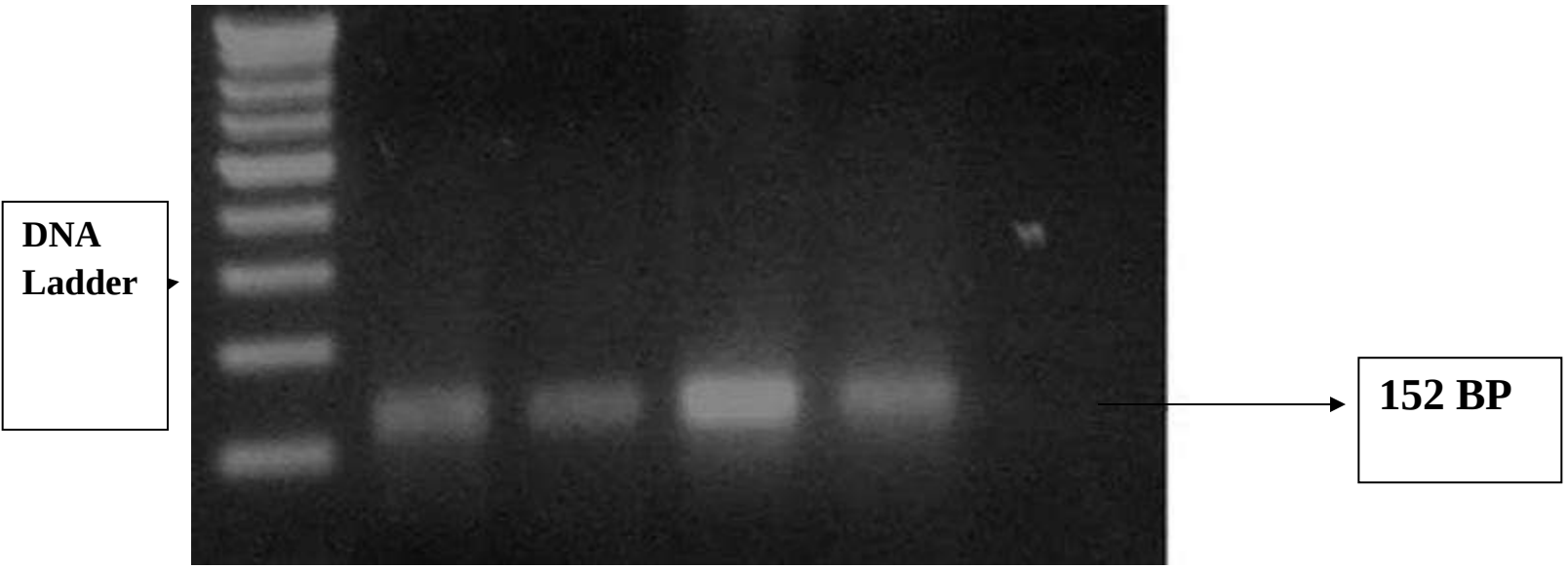

Fig No. 8: The Gene Exctraction ExoT gene:

L corresponds to the DNA Ladder; L1- L2 are the sample positive for ExoT gene; L3 corresponds to the Positive Control for ExoT gene; L4 corresponds to the sample positive for ExoT gene; L5 is the Negative Control gene for ExoT gene

DISCUSSION

Pseudomonas aeruginosa is a significant cause of human infection, particularly in hosts with weak defence mechanisms. It is a frequent nosocomial pathogen known for its multidrug resistance (MDR) and potentially fatal infections in critically unwell patients. Carbapenems are increasingly being used as a last resort antibiotic treatment for serious infections caused by MDR P. aeruginosa [27].

In the current study a total of 400 clinically suspected cases from both IPD & OPD were included out of which 130 were culture positive for *Pseudomonas* infection. Therefore, the prevalence rate of *Pseudomonas aeruginosa* was found to be 32.5%. This study was similar to the study performed by the other author where the prevalence of *Pseudomonas aeruginosa* was found to be 33.8% [28].

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In India, prevalence rate of *P.aeruginosa* infection varies from 10.5% to 30%. There was a another study conducted by [29] where the prevalence was found to be 2.76% which was in contrast to our study.

From the present study it was observed that the Males 84 (64.6%) were more affected with the infection as compared to the Females 46 (35.3%). There was another study which was parallel to our study where the ratio of males being affected was more as compared to the females where 80 (66.6%) were from males and 40 (33.3%) were from females [30]. There were other studies performed by the other research investigator which were in support to the present study where the Male preponderance was found to be 55.76% [28]. Similar observations were made by (Anupurba S *et al,* 2006) [31]. Outdoor activity, personal habits, nature of work and exposure to soil, water and other areas which are inhabited by organism could be the reason for male preponderance. It was also noted that the age group of 21-30 years of age followed by 31-40 was affected the most. In the age group of 0-10 years and above 71 years was the least affected with the *Pseudomonas aeruginosa* infection. This was in accordance with other study reported by OkonK O *et al, 2009* (45.88%) [32] , where the common age group was between 21–40 years of age. It was also observed that the maximum number of isolates observed were from the Urine sample (49.2%) followed by the pus (24.6%) and sputum (14.6%), Et- secreation (6.1%) and throat swab (3.8%), and least was from the blood (1.5%). Similar results had been obtained in different studies in India reported by (Chander Anil *et al,* 2016) [33] , (Mohanasoundaram *et al*, 2011) [34] (Arora, D *et al,* 2011) [35] . A similar observation was reported in another study (Agarwal S *et al*, 2017) [36]. Most of the other studies showed the highest isolation from pus specimens (Bezalwar PM *et al,* 2019) [37] and Charde VN *et al,*2019) [38] (Koirala A *et al* ,2017) [39] . The high rate of isolation from the urine samples in our study may be due to its ability to cause urinary tract infections in most people (Forbes BA *et al,* 2007) [40].

It was observed that the Sensitivities of Colistin was (96.6%), Piperacillin-tazobactam (77.6%), Amikacin (77.6 %), and cefepime (74.6 %) were found to be the most effective Antibiotics. The resistance to ciprofloxacin was (46.1%), Levofloxacin (53.8%), Gentamicin(62.3%), Imipenem (67.6%), Tobramycin(68.4%), Ceftazidime (68.4%).

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This study was similar to the study performed by (Nabina Maharjan *et al,* 2022) [41] where , polymyxin B followed by imipenem were the two most effective drugs showing 92.6% and 89.7% sensitivity respectively. Ceftazidime was found to be the least effective drug showing 44.1% sensitivity. There studies also supported our work where similar results was found (Kaur A and Singh S *et al, 2*018) (Shrestha P *et al,* 2018) [42,43].

In the present study it was observed that 67 (51.5%) of the *Pseudomonas aeruginosa* isolates showed biofilm formation. The present study was in support to the results of other studies ,(Pournajaf A *et al,* 2018) (Vasiljević Z *et al,* 2014*)* where a significant number of included isolates (83.75%) formed biofilm [44,45].

P. aeruginosa secretes four recognised effector proteins through its type III secretion system: exoS, exoT, exoU, and exoY [46]. These proteins influence host cell processes involved in cytoskeletal organisation and signal transmission [47]. ExoS and exoT are bifunctional toxins that activate ADP-ribosyltransferase and GTPase, respectively [48]. ExoT exhibits lesser ADP-ribosyltransferase activity than exoS [48]. The majority of P. aeruginosa strains include exoT and exoY genes; however, the presence of exoS and exoU varies significantly among isolates and appears to be mutually exclusive [47].

In the present study it was observed that out of 130 Pseudomonas isolates there were 52 (40%) which expressed ExoT gene. This study was in support with the study by Dadmanesh et al. [49, 50] where *exoS* and *exoT* rate as 73.91% and 69.21% respectively.
There was another study which was in contrast to the present study where the rate of Exo T was 5% and exo U was 52%[51]. Another study stated that exoT gene occurred in 20 (66.67%) isolates of *P. aeruginosa*, while 10 (33.33%) showed negative amplification results [52].
In other similar study in southern India, the prevalence of exoT was recorded as 84% [53]. 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

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world. Prevalence of exoT gene recorded in Iran was 36.27% [54] and in Egypt and Romania, prevalence of exoT in P. aeruginosa clinical isolates were recorded as 100% [55,56].

The results of the present investigation shows that the distribution of exo genes varied across *P. aeruginosa* clinical isolates from Uttar Pradesh.

ExoT virulence genes have been shown to play a significant role in *P. aeruginosa* pathogenicity, which is important for understanding the prognosis of pseudomonas infections and developing a vaccine that will effectively prevent them. Biofilm formation has been identified as a significant factor of pathogenicity in P. aeruginosa infections . Biofilm development exacerbates persistent bacterial infections and lowers antimicrobial medication efficacy [57] .

CONCLUSION

Finding virulent pathogen indicators to detect acute and chronic infections early on remains a critical field that demands further exploration. These studies and study findings aid in the prevention of bacterial infections and can be quite beneficial in the management of Pseudomonas infections. In addition to identification and antimicrobial susceptibility tests, future diagnostic microbiology trends should focus on the development of quick tests for detecting virulence factors, which are essential epidemiological markers.

Declarations:

Conflicts of interest: There is no any conflict of interest associated with this study

Consent to participate: We have consent to participate.

Consent for publication: We have consent for the publication of this paper.

Authors' contributions: All the authors equally contributed the work.

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