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GENOTYPIC DETECTION OF SOME pSEUDOMONAS AERUGINOSA VIRULENCE GENES AMONG DIFFERENT CLINICAL ISOLATES

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ABSTRACT

Background: Pseudomonas aeruginosa an opportunistic pathogen that can infect virtually all tissues via a number of virulence factors and possess a variety of antibiotic resistance mechanisms.

The aim of this study was molecular identification of genus and species level of P. aeruginosa by detection of oprI, oprL genes respectively by PCR. Determination of antibiotic resistance profile among isolates, as well as, detection of virulence genes toxA, plcH, plcN and LasB by PCR.

Identification and antimicrobial susceptibility of fifty P. aeruginosa isolates obtained from different clinical specimens were performed on VITEK-2 Compact system. PCR was used for the molecular confirmation of genus and species level of P. aeruginosa by detection of oprI, oprL genes, as well as, the molecular detection of virulence genes toxA, plcH, plcN and LasB.

The highest prevalence of resistance was detected against 3rd and 4th generation cephalosporins (80%). Resistance to other β-lactam antibiotics including piperacillin-tazobactam, monobactams, imipenem and meropenem was 62%, 70%, 64% and 62% respectively. Resistance to aminoglycosides ranged from 44% to amikacin and 56% to tobramycin. High level of resistance (68%) to fluoroquinolones was detected. All isolates showed susceptiblity to lipopolypeptide antibiotics, colistin and polymixin. Using multiplex PCR, all strains were confirmed molecularly as P. aeruginosa via detection of oprI, oprL genes in the 50 (100%) isolates, plcH and plcN were detected in all 50 (100%) of strains and toxA in 46 (92%) and LasB in 50 (100%) of strains each in separate PCR reactions.

This investigation is a thorough report of some virulence factors and antibiotic resistance properties of P.aeruginosa isolated from Egyptian human clinical samples. Results affirm that all tox A, lasB, plcH, plcN genes are prevalent in human infections. Antibiotic resistance was high. Hence, judicious antibiotics usage is required by clinicians.

INTRODUCTION

Pseudomonas aeruginosa is a motile, non-fermenting,Gram-negative bacteria from Pseudomonadaceae family. [(1)]\ It is an opportunistic pathogen capable of infecting almost all tissues, and responsible for hospital acquired infections[(2)].

Immunocompromised patients, burn patients, mechanically ventilated patients, leukemic and cystic fibrosis patients are particularly susceptible to *P. aeruginosa* infections[(3)].

The critical attributes responsible for the pathogenic potential of *P. aeruginosa* are the production of a multitude of virulence factors, biofilm formation and antibiotic resistance[(4)]. These factors enable successful infection and colonization across a wide range of environments. This arsenal includes; elastase, phosphor-lipase C (PLC)[(5)], alkaline protease, exotoxin A, exoenzyme S, pyocyanin, pyoverdin, hydrogen cyanide, as well as cell-associated factors, such as alginate, lipopolysaccharide, flagella, pili[(6, 7)].

Exotoxin A is the most toxic substance in *P. aeruginosa*, it catalyzes the ADP ribosylation of the eukaryotic elongation factor 2 (eEF-2), and thus significantly affects protein synthesis of the host cells[(8)].

LasB elastase has an elastolytic activity on lung tissue. It is a zinc metalloprotease encoded by *lasB* gene [(9)]. Moreover, the phospholipids in pulmonary surfactants may be hydrolysed by PLC-H and PLC-N, two phospholipases C encoded by *plcH* and *plcN* respectively[(10)].

The outer membrane proteins of *P. aeruginosa*, OprI and OprL, are of major importance in the interaction of the bacterium with the surrounding and the inherent resistance of *P. aeruginosa* to antimicrobial agents [(11)] As they are only detected in this organism, they are a reliable factor for rapid molecular identification of *P. aeruginosa* in clinical samples[(12)]. Although conventional microbiological techniques for identifying *P. aeruginosa* are dependable, they require a number days to be completed. Rapid identification of isolates is critical for subsequent treatment decision of patients. PCR has the potential for rapid identification of microbial species by amplification of unique sequences in a particular organism[(6)].

Therefore, this study aimed for molecular identification of genus, species levels of *P. aeruginosa* by

detection of *oprI*, *oprL* genes respectively by PCR. Determination of antibiotic resistance profile among isolates, as well as, detection of virulence genes *toxA, plcH, plcN and LasB* by PCR.

Subjects and Methods:
Fifty *P. aeruginosa* isolates were obtained from several clinical specimens after the approval of ethical committee of the Medical Research Institute, Alexandria University.

Bacterial isolation, identification and storage:
All specimens were cultured routinely on blood and MacConkey's agar. After overnight incubation, Gram negative, non-lactose fermenting oxidase positive colonies were further identified using VITEK-2 Compact system (GN-13 card) (bioMerieux, France). Isolates were stored in Luria Bertani [13] broth containing 30% glycerol and tubes stored at -80°C. For bacterial revival, one loopful was streaked over blood agar and incubated at 37°C.

Antimicrobial Susceptibility testing of *P.aeruginosa* isolates:
Antimicrobial susceptibility was performed on VITEK-2 Compact system (bioMerieux, France) according to manufacturer's instructions. The AST-NO21 cards and software were used for analysis.

Polymerase chain reaction (PCR):
PCR was used for the confirmation of genus as well as species level of *P. aeruginosa* by detection of *oprI*, *oprL* genes, moreover, detection of virulence genes *toxA, plcH, plcN and LasB.*

DNA extraction was performed by boiling method[14]. Briefly, 2 to 3 colonies from overnight cultured were suspended in TE buffer containing 0.1% triton X100. Bacterial suspensions were incubated in a boiling water bath for 15 minutes followed by rapid cooling on ice. After centrifugation for 15 minutes at 14,000 rpm in a microfuge, the supernatant was served as a source for bacterial DNA.

PCR was carried out in 25 µl final volume containing 12.5 µl hot start PCR master mix MyTaq^{TM} HS Red Mix (BioLine, London, UK), 10 pmole of each primer (all primers were purchased from Thermo Fisher Scientific, California., USA) (Table I) and 0.5 µl bacterial DNA. A negative control was prepared by the addition of the same contents to the tube with water instead of the extract. All PCR reactions were performed on Veriti thermal cycler (Applied Biosystems, California, USA). *oprI*/*oprL* and *plcH*/*plcN* were detected in 2 multiplex PCR reactions, while *toxA* and *lasB* were detected using singleplex PCR. The reactions were performed according to the following thermal profile, initial denaturation 95ºC for 3 minutes followed by 40 cycles of denaturation at 95ºC for 30 seconds, annealing for 15 seconds and extension at 72ºC for 1 min/kb followed by final extension at 72ºC for 5 minutes. PCR products were separated by gel electrophoresis on 2% agarose gel containing 0.5 µg/ml ethidium bromide

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Table (1): Primers used for the PCR reactions

Primer	Nucleotide Sequence 5' – 3'	Annealing temperature °C	Amplicon size (bp)	Reference
oprI- F	CGTTCTGAAATTCTCTGCTATGAACAA	55	249	(15)
oprI-R	CTTGCGGCTGGCTTTTTCCAG			
oprL- F	ATGGAAATGCTGAAATTCGGC	55	504	(16)
oprL-R	CTTCTTCAGCTCGACGCGACG			
plcH- F	GAAGCCATGGGCTACTTCAA	55	307	(17)
plcH -R	AGAGTGACGAGGAGCGGTAG			
plcN - F	GTTATCGCAACCAGCCCTAC	55	466	
plcN -R	AGGTCGAACACCTGGAACAC			
toxA - F	GGTAACCAGCTCAGCCACAT	56	352	
toxA -R	TGATGTCCAGGTCATGCTTC			
lasB- F	GGAATGAACGAAGCGTTCTC	55	300	
lasB-R	GGTCCAGTAGTAGCGGTTGG			

RESULTS

The 50 *P. aeruginosa* isolates were isolated from 27 (54%) females and 23 (46%) males. Twenty (40%) isolates were from respiratory tract infections, 15 (30%) from wound infections, 8 (16%) from bloodstream infections, and 7 (14%) from urine samples.

Molecular diagnosis of *P. aeruginosa:*

Using multiplex PCR, all strains included in the current study were confirmed molecularly as *P. aeruginosa* via detection of *oprI*, *oprL* genes in the 50 (100%) isolates.

Molecular detection of *P. aeruginosa* virulence genes:

Using multiplex PCR, *plcH* and *plcN* were detected in all 50 (100%) of strains and *toxA* in 46 (92%) and *LasB* in 50 (100%) of strains each in separate PCR reactions. *ToxA* gene was not detected in 2 samples from bloodstream infections, 1 from respiratory tract infection and 1 from wound infection.

Table (18) shows the antimicrobial susceptibility testing of the 50 *P. aeruginosa* isolates included in this study. The highest prevalence of resistance was detected against third and fourth generation cephalosporins (80%). Resistance to other β-lactam antibiotics including piperacillin-tazobactam, monobactams, imipenem and meropenem was 62%, 70%, 64% and 62% respectively. Resistance to aminoglycosides ranged from 44% to amikacin and 56% to tobramycin. High level of resistance (68%) to fluoroquinolones was detected. All isolates were susceptible to lipopolypeptide antibiotics, colistin and polymixin.

Table (III) shows the distribution of antimicrobial resistance among clinical samples. Resistance showed lower prevalence in urinary tract infections in comparison to other sites of infection. Additionally, resistance to 3rd and 4th generation cephalosporins recoded the highest prevalence among all infection sites.

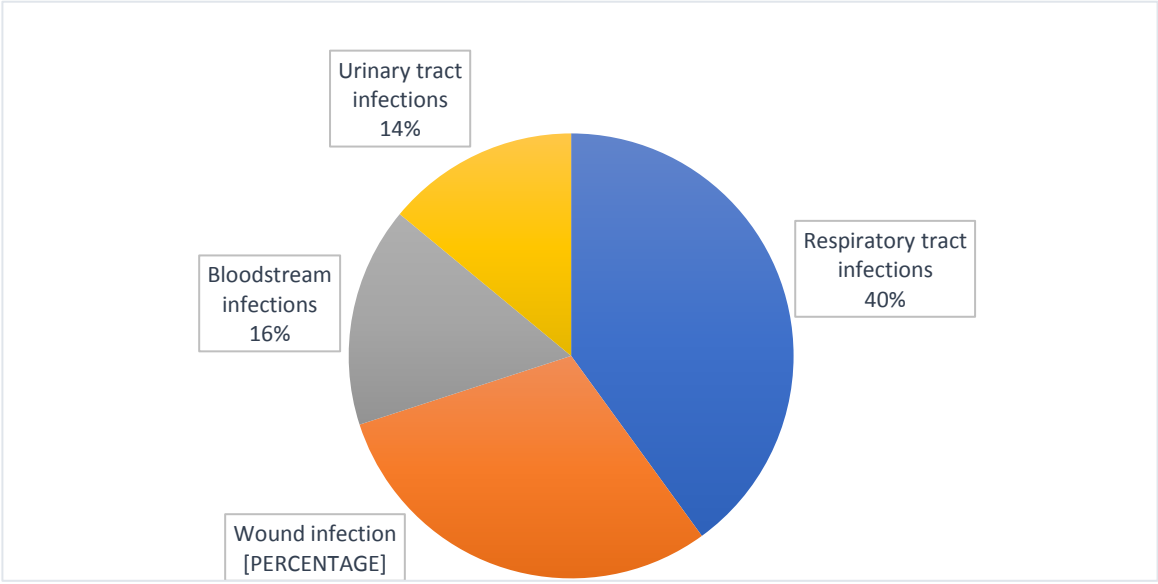

Figure (1): Distribution of the studied cases according to the site of isolation

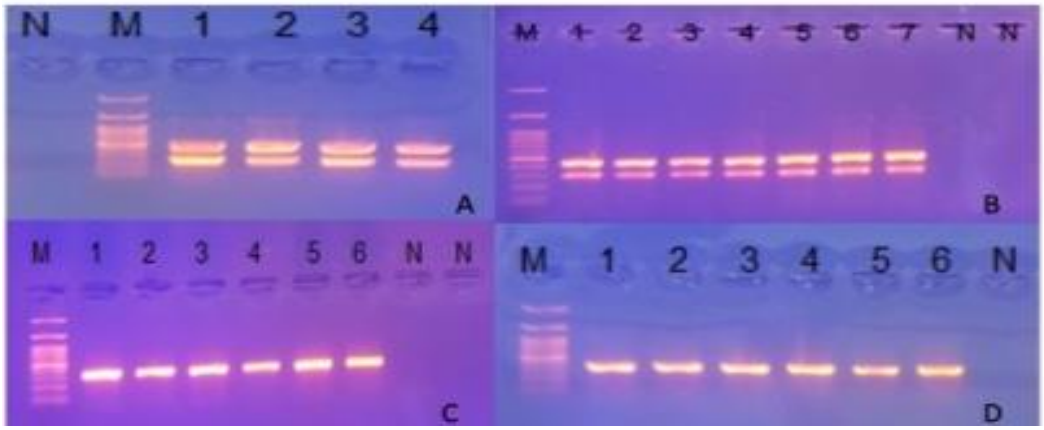

Figure (2): Ethidium bromide stained agarose gel showing the expected size of the amplified band of **A**=*OprI* 249 bp and *OprL* at 504 bp in lanes 1-4, B=PlcH at 307 bp band size and PlcN at 466 bp in lanes 1-7, C= LasB in lanes 1-6 at 300 bp and D= toxA at 352bp in lanes 1-6. M is100bp DNA ladder. N shows the lanes where water replaced the template in the PCR as a negative control.

Table (2) Antimicrobial susceptibility testing of the 50 P.aeruginosa isolates

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Piperacillin-Tazobactam	15	30	4	8	31	62
Ceftazidime	8	16	2	4	40	80
Cefipime	9	18	1	2	40	80
Aztreonam	11	22	4	8	35	70
Imipenem	18	36	0	0	32	64
Meropenem	19	38	0	0	31	62
Gentamicin	22	44	2	4	26	52
Tobramycin	21	42	1	2	28	56
Amikacin	27	54	1	2	22	44
Ciprofloxacin	16	32	0	0	34	68
Levofloxaxin	16	32	0	0	34	68
Colistin	50	100	0	0	0	0
Polymixin	50	100	0	0	0	0

Table (3): Antimicrobial susceptibility testing of the 50 P.aeruginosa isolates

	Respiratory tract		Wound swab		Blood		Urine		Total	
	N=20	%	N= 15	%	N= 8	%	N=7	%	N= 50	%
Piperacillin-Tazobactam	15	75	7	47	6	75	3	43	31	62
Ceftazidime	18	90	10	68	7	87.5	5	71	40	80
Cefipime	18	90	10	68	7	87.5	5	71	40	80
Aztreonam	16	80	10	68	5	87.5	4	57	35	70
Imipenem	15	75	8	53	6	75	3	43	32	64
Meropenem	15	75	8	53	5	62.5	3	43	31	62
Gentamicin	13	65	6	40	4	50	3	43	26	52
Tobramycin	14	70	6	40	5	62.5	3	43	28	56
Amikacin	11	55	6	40	3	37.5	2	21	22	44
Ciprofloxacin	16	80	9	60	6	75	3	43	34	68
Levofloxaxin	16	80	9	60	6	75	3	43	34	68
Colistin	0	0	0	0	0	0	0	0	0	0
Polymixin	0	0	0	0	0	0	0	0	0	0

DISCUSSION

P.aeruginosa as an opportunistic pathogen has different virulence factors which aid the bacteria to colonize different niches in their host and the bacteria are a leading cause of nosocomial and community-acquired infections worldwide[19]. At present, bacterial culture remains the most commonly used method for detecting *P. aeruginosa*, but it is time-consuming and susceptible to conflicting results due to sample contamination. Also, this method has low sensitivity. Because of the significance of early diagnosis, direct detection of *P. aeruginosa* in clinical samples will reduce diagnostic turnaround time and minimize the risk of contamination[20].

Identification of *P. aeruginosa* traditionally relies on phenotypic methods that still is the most accurate method. Biochemical testing takes long time to perform and requires extensive hands-on work, both for setup and for interpretation. Molecular methodologies have been reported to be superior to the phenotypic methods for identification of *P. aeruginosa*[12, 21] . A designed multiplex PCR assay based on *oprI* and *oprL* genes for molecular confirmation of *P. aeruginosa* revealed that the specificity and sensitivity of PCR were 74 and 100%, respectively**.** The main advantage of multiplex PCR is its ability to simultaneously amplify multiple PCR products in a single reaction, thereby enabling multiplex detection and significantly reducing the detection cost and time requirements[22]. Lavenir et al [23] also stated that all of *P. aeruginosa* strains contained the *oprI* and *oprL* genes. Similarly El-Sheshtawy et al [24] in their study reported that all of the 30 isolates were positive for both genes. This is consistent with this study result as both genes *oprI*, *oprL* were detected in all 50 (100%) samples.

P. aeruginosa is capable of inducing different types of infections in humans. In the present study, twenty (40%) *P. aeruginosa* isolates were obtained from respiratory tract infections, 15 (30%) from wound, 8 (16%) from bloodstream, and 7 (14%) from urine tract infections.\

Viedma *et al*[25] in their study showed that, 36 (19.7%) suffered from lower respiratory tract infection, 30 (16.4%) patients with urinary tract infection, 28 (15.3%) patients with bloodstream infection and 22 (12%) patients with intra-abdominal infection out of 183 patients. The high incidences of *P. aeruginosa* in respiratory infections have been previously reported in cystic fibrosis patients [26-28].

The treatment of *P. aeruginosa* infections is significantly challenging. The diversity of antibiotic resistance mechanisms contributes to the development of multidrug-resistant strains that make conventional antibiotics ineffective for therapy [29].

In this study, antimicrobial resistance showed lower prevalence in urinary tract infections in comparison to respiratory, bloodstream and wound infections which may be attributed to community-acquired origin of infection. Additionally, resistance to third and fourth generation cephalosporins recoded the highest prevalence among all infection sites.

Fazeli *et al*[30] in 2014 stated that their bacterial isolates were multidrug resistant. Resistance to gentamicin and ciprofloxacin was reported in 39.21% and 21.56% of their bacterial strains isolated from hospital infections. In another study by Fazeli *et al*[31] including hospital acquired infections, hospital means and personnel's specimen, they showed that 29% and 32.2% of the *P. aeruginosa* strains were resistant to ciprofloxacin and gentamicin, which was much lower than our results. Lower resistance rates were reported in Latin America 26.8% [32] and 10%-32% in Europe[33, 34].

In a study of Viedma *et al*[25], all the isolates were only susceptible to colistin (100%) and amikacin (75%). In this study colistin was also sensitive in all 100% of isolates.

The antibiotic resistant pattern found by Akingbade *et al* [35] investigation showed that *P. aeruginosa*, had low resistant to ceftazidime (20%), gentamicin (26.4%), levofloxacin (30.9%), ceftriaxone (34.5%), and ciprofloxacin (35.5%), which was also much lower than our results. Similar results have been reported by Lim *et al*[36] from Malaysia and Smith *et al* [37] from Nigeria.

Pathogenicity of *P. aeruginosa* is multifactorial. LasB is one of the most important proteases of *P. aeruginosa*[38], which causes direct injury to the lung early in the disease. Bleeding in the lung is often seen with pulmonary infections by *P. aeruginosa* due to the elastin breakdown in the blood vessels walls [39]. In this study, *lasB* gene was detected in all isolates. Nikbin et al[40] in their study stated that all isolates harbored *lasB* gene. This finding is in agreement with reports by Nicas *et al*[41] and Lomholt *et al*[38]. Mutation of *lasB* gene reduces *P. aeruginosa* invasion remarkably. Prevalence of the *lasB* gene in all clinical isolates highlights its importance to of *P. aeruginosa* survival in various conditions[42].

Toxin A has distinct role in hindering wound contraction and remedial [43]. Hummel and Unger[44] established the first PCR method that detected *P. aeruginosa* based on the toxin A gene and evaluated its efficacy in mechanically ventilated patients. The results showed that the method detected 57 positive samples out of 364 total samples, whereas the conventional culture method only detected 36 positive samples, indicating that the toxin A gene-based PCR method had higher sensitivity. Using PCR, we detected toxinA gene in 100% of our isolates.

Phospholipase C plays a role in the lysis of target cells (pulmonary atelectasis) and is involved in acute and chronic infection[45]. Most of the 151 isolates in a study performed by Elogne et al[10] detected the virulence genes; *plcH* in84.1% and *lasB* in 72.8%. The *plcH* gene was found in all types of infections with prevalence ranging from 66.7% to 84.6%. The highest prevalence was found in sepsis with 84.6% followed by lung infections with 81.5% and then urinary tract infections in 78.8%. This

frequency at the urinary level is close to that Heidary *et al.* 70.4% in 2016 in Iran [46]. Elastase causes the destruction of tissues containing elastin, tissue necrosis and haemorrhage[10]. The *lasB* gene was found in isolates from all infectious sites with variable rates. Prevalence were high and ranged from 57.1% to 81.5%. The highest rate was found at the pulmonary level (81.5%). These results are close to those of Mitov I. *et al*[47] with *plcH* in (91.6) and *lasB* in (100%)[47]. Similarly, in this study *plcH* and *lasB* were detected in all 100% of isolates.

On the other hand, the PCR results of virulence factor in the study by Al-Dahmoshi *et al* [48] showed that exoA was present only among 12(46.15%), oprL was 11(42.3%), oprI was 22(84.61%) and lasB was 18(69.23%).

Coexistence of ExoA responsible for toxigenesis and LasB responsible for invasiveness makes both of mechanism of infection available and delays wound healing[49-52]. Coexistence of more than one virulence factor in the same isolate were recorded and the results displayed that 8/26 have all five factors, 4/26 have four, 1/26 have three and 5/26 have only two virulence factors [48]. In this study, 46/50 samples had 4 virulence factors and only 4/50 had only 3 virulence factors, namely, plcH, plcN and las B.

Nikbin *et al.* in 2012 stated that all isolates carried oprI, oprL and lasB genes[40]. The presence of ExoA, OprL, OprI, LasI and LasB among P. aeruginosa isolates propose their association with different levels of virulence and pathogenicity[48, 53].

In summary, the study provides an insight into the phenotypic and genotypic characteristics of P. aeruginosa emerged in Egypt. Our finding highlighted a high rate of resistance to antibiotics. In order to reduce the risk of spread of highly resistant strains, we suggest the need to establish a periodic surveillance system, to enhance infection prevention and control measures in healthcare setting, and to increase awareness among physicians and the general public about the rational use of antibiotics. Future work should target mechanisms of resistance and genetic diversity of P. aeruginosa strains to draw more far-reaching conclusions.

Further studies will be useful to affirm the correlation between the drug resistance pattern and virulence factor expression.

This study is a thorough report of virulence factors and antibiotic resistance properties of P. aeruginosa isolated from Egyptian human clinical samples. Our results disclosed that all tox A, lasB, plcH, plcN genes are prevalent in human infections.

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