RESEARCH ARTICLE

Molecular detection of virulence genes in pseudomonas aeruginosa isolated from different clinical infections

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Abstract

Objective To isolate *pseudomonas aeruginosa* from different clinical specimens, and to detect virulence factors.

Method: The cross-sectional study was conducted at Al-Zharr Hospital and private clinics in Kut city of Wasit province in Iraq from October 15, 2021, to April 30, 2022, and comprised samples obtained from different patient sites, such as wound swabs, ear swabs, urine samples, burn swabs and respiratory tract swabs. The samples were subjected to molecular testing using, among other tools, polymerase chain reaction. The isolates were cultured on different media, such as blood agar, MacConkey agar and cetrimide agar, and were further diagnosed by biochemical testing. Data was analysed using SPSS 20.

Results: Of the 212 samples, there were 70(33%) wound swabs, 53(25%) ear swabs, 41(19.3%) urine samples, 33(15.6%) burn swabs and 15(7%) respiratory tract swabs. Pseudomonas aeruginosa was identified in 54(25.47%) isolates via culture-based characterisation. All 54(100%) isolates were positive for mexR and toxA on polymerase chain reaction, 44(81.48%) were positive for exoU gene, 43(79.62%) for exoT gene, 36 (66.66) for exoT gen, 35(64.81%) for plcH, gene and 14(25.92 %) for exoS gene.

Conclusions: Wound swabs were found to be the most common site for *pseudomonas aeruginosa*, followed by ear swabs and urine samples.

Keywords: *Pseudomonas aeruginosa*, Virulence, Agar, Cetrimonium, Virulence, Burns, Polymerase chain reaction, DNA.

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Introduction

Pseudomonas (P.) aeruginosa is a gram-negative bacillus belonging to the Pseudomonadaceae bacterial family. It has a diameter of around 1mm (1.5-3.0 x 0.5m) in length, is motile due to one or more polar flagella, and is non-spore-forming and non-capsulated.[1] *P. aeruginosa* is a strict aerobe and these aerobes can be found as single cells, in pairs, or as short chains.[2] As it is commonly found in the environment and can cause a wide range of illnesses in humans, animals and plants, *P. aeruginosa* is regarded as a significant bacterial species. Additionally, microorganisms that are thought to be a cause of nosocomial infections are more significant.[3] *P. aeruginosa* causes several diseases in the human body, such as infections of the skin, burns, eyes, urinary tract, otitis media, bacteraemia as well as bones and joints.[4] It can secrete many toxic proteins that are believed to act as virulence factors; these proteins can cause extensive tissue damage and result in invasion of the bloodstream.[5] The exotoxin A (exoA), which shares the same mechanism of action as the diphtheria toxin, is a crucial component of the pathogenicity of *P. aeruginosa*. Localised tissue injury, bacterial invasion and immunosuppression are all caused by exoA.[6]

P. aeruginosa is naturally resistant to several antibiotic agents due to mutational changes or via the acquisition of genetic materials that provide resistance. The appearance of multidrug resistant (MDR) strains can occur as a result of the selective pressure caused by anti-microbial therapies, and the acquired multidrug resistance to many antibiotics results in an increased morbidity and mortality rate following infection.[7,8] Due to decreased membrane permeability, anti-microbial agent efflux pumps and acquired susceptibility by the development of resistance genes, *P. aeruginosa* has a high level of anti-microbial drug resistance in addition to its virulence factors that also contribute towards drug resistance.[9] The rise of MDR P. aeruginosa poses a public health risk by limiting the number of effective antibiotic treatments commercially available. Antibiotic-resistant bacteria are on the rise across the world, posing a serious threat of treatment failure.[10] The current study was planned to isolated *P. aeruginosa* and to detect various virulence factors.

Materials and Methods

The cross-sectional study was conducted at Al-Zharr Hospital and private clinics in Kut city of Wasit province in Iraq from October 15, 2021, to April 30, 2022. approval was

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obtained from Wasit Health Director to conduct the study

Using simple random sampling technique, patients of either gender were enrolled for obtaining specimens from wound swabs, ear swabs, urine samples, burn swabs and respiratory tract swabs. The sample size was calculated using the formula:[11]

$$\frac{Z_{1-\alpha/2}{}^2 p^{(1-p)}}{d^2}$$

Standard normal variate $Z_{1-\alpha}/2$ was 1.96 at 5% type 1 error (p=0.05) and 2.58 at 1% type 1 error (p=0.01). As p<0.05 are typically regarded as significant in research, 1.96 was utilised in the formula in which p was the population expected proportion based on a previous study,[12] and d was the absolute inaccuracy or precision determined by the researchers.

Cotton swabs were used to take samples from the patients. Once obtained, they were cultured on several different media, such as blood agar, MacConkey agar and cetrimide agar, the latter being a selective medium for P. aeruginosa. The samples were incubated at 37°C for 24 hours after which cultured bacteria were isolated and identified according to colony morphology, shape, size, colour and pigment production.

Deoxyribonucleic acid **(**DNA) extractions were carried out using a commercial kit (Presto™ Mini gDNA Bacteria Kit, Geneaid, Thailand) to obtain DNA templates for use in PCR assays. The DNA of *P. aeruginosa* isolates was extracted as per the manufacturer's instructions.

For cell harvesting pre-lyses, the bacterial strains were cultured on cetrimide agar for 18 hours at 37°C. Then, they were harvested by centrifugation for 1 minute at a speed of 14,000rpm, with the supernatant being discarded.

Further, 20µL of proteinase potassium (K) (solution and 180µL of buffer Guanidinium thiocyanate (GT) were added to the pellet and mixed, with the sample tubes inverted every 3 minutes for the duration of the incubation period.

After mixing for 10 seconds with 200µL of buffer Guanidine Brochloride (GB), the cell lysate was incubated for 10 minutes at 70°C, with sample tubes mixed by inversion every 3 minutes to induce lysis. The elution buffer was pre-heated 200 l/sample at 70°C for DNA elution.

For DNA binding, the lysate samples were treated with 200µL of 100% ethanol and thoroughly mixed by shaking. The mixture was transferred to a spin column in a 2ml collection tube, and placed in a new 2ml collection tube for the genome DNA (GD) column.

For DNA elution, the spin column was placed in a 1.5 microcentrifuge tube, and 100µL of pre-heated elution buffer was added to the middle of the column matrix. After letting the mixture stand for 3 minutes to ensure that all of the elution buffer had been absorbed, the spin column was centrifuged for 30 seconds at 14,000rpm to elute the purified DNA. The extracted DNA was stored in the freezer at -20°C until use.

The concentration and purity of the DNA was measured by using an instrument (Nano Drop) and agarose gel electrophoresis.

During the process, 1µl of the extracted DNA was added to the instrument in order to detect DNA concentration and purity by analysing the optimal degree) OD((260/280 ratio to verify the protein and DNA concentration.

For agarose gel electrophoresis, 1x Tris-borate-EDT(TBE) buffer was placed in the electrophoresis tank, after which the agarose tray was immersed in the electrophoresis tank. It was ensured that the buffer was roughly several millilitres above the agarose surface. Each well was filled with 5µl of the sample and 2µl of dye, and the tank was then filled and closed. Electrophoresis was performed using 70 volt/cm of gel run swat electrophoresis. With the use of gel paper, the agarose was extracted from the tank and visualised.

Optimization of the PCR primers was done via temperature gradients. Temperature gradient PCR assays are reactions performed using different temperatures of annealing. This assay was performed to identify the optimum annealing temperatures of the various primers that were used to detect *P. aeruginosa*.

For the optimization of the primers used, 2.5µl of the master mix was mixed with 5-6µl of DNA, along with 1µl of the forward and revers primers. Optimisation was programmed for enterotoxin A (toxA), enterotoxin S (exoS), enterotoxin Y (exoY), enterotoxin U(exoU), enterotoxin T(exoT), multi-drug resistance (mexR) and haemolysinphosphlipase (plcH), and primer of gene grades were chosen, and the annealing temperature of PCR were set at 55°C, 58°C and 52°C.

Detection of toxA, exoS, toxY, toxU, toxT, mexR, plcH genes was carried out by mixing 12.5ml master mix, 5-6ml DNA, 1ml each of forward and reverse primers, and nuclease-free deionised water to a final volume of 20ml, as per the manufacturers' instructions.

PCR cycling programme parameters used in the reaction for the detection of the genes of interest were noted (Tables 1-3).

Data was analysed using SPSS 20. Chi-square test was used

to analyse the data. P<0.001 was considered statistically significant.[12]

Results

Of the 212 samples, there were 70(33%) wound swabs, 53(25%) ear swabs, 41(19.3%) urine samples, 33(15.6%) burn swabs and 15(7%) respiratory tract swabs. *Pseudomonas aeruginosa* was identified in 54(25.47%) isolates via culture-based characterisation. *P. aeruginosa* was found in wound infections 23(32.85%), followed by 13(24.52 %) ear infections, 11(26.82 %) urinary tract infections (UTIs), while the lowest incidence was 2(13.3%) in respiratory tract infections (RTIs) (Table 4).

P. aeruginosa was identified in 54(25.47%) isolates. All 54(100%) isolates were positive for mexR, and the PCR product was 224bp in size. Also, all 54(100%) isolates were positive for toxA, and the PCR product was 190bp in size.

Further, 44(81.48%) isolates were positive for exoU, and the PCR product was 507bp in size, and 43(79.62%%) exoT, with PCR product size 489bp, 36(66.66) isolates were positive for exoY. Among the isolates, 35(64.81%) were positive for g plcH ene, and the PCR product was 224bp in size, while 14(25.92%) isolates were positive for ExoS, and the PCR product was 289bp in size.

Prevalence of virulence genes in the *P. aeruginosa* isolates significantly different in terms of clinical samples (p<0.05) (Table 5).

Table-1: Thermal cycling programme for toxA and plcH genes.

No	Steps	Tem (°C)	Time	Cycles
1	Initial Denaturation	94	5min	1
2	Denaturation	94	30sec	35
3	Annealing	58	50sec	35
4	Extension	72	1min	35
5	Final extension	72	10min	1
6	Holding	4	∞	1

Table-2: Thermal cycling programme for exoT and exoY genes.

No	Steps	Tem (°C)	Time	Cycles
1	Initial Denaturation	94	5min	1
2	Denaturation	94	30sec	35
3	Annealing	58	30sec	35
4	Extension	72	1min	35
5	Final extension	72	5min	1
6	Holding	4	∞	1

Table-3: Thermal cycling programme for mexR and exoS genes.

No	Steps	Tem (°C)	Time	Cycles
1	Initial Denaturation	95	5min	1
2	Denaturation	94	30sec	35
3	Annealing	55	30sec	35
4	Extension	72	7min	35
5	Final extension	72	5min	1
6	Holding	4	∞	1

Table-4: Prevalence of *pseudomonas (P.) aeruginosa* isolates in the clinical samples.

Clinical Samples	Total	Positive samples	Negative samples
Wound	70	23 (32.85)	47 (67.14)
Ear	53	13 (24.52)	40 (75.47)
Uti	41	11 (26.82)	30 (73.17)
Burn	33	5 (15.15)	28 (84.84)
Respiratory tract	15	2 (13.33)	13 (86.66)
Total	212	54 (25.47)	158(74.53)
X^2		5.09 (NS)	
p-value		0.472*	

NS: Non-significant. * Not significant (p>0.05).

Table-5: *Pseudomonas (P.) aeruginosa* distribution according to isolation sources.

Clinical Samples	Total	Exo T	exo S	exo Y	exo U	toxA	Mex R	Plc H	X^2	*p*-value
Wound	23	20	7	19	21	23	23	16	51.24	0
		(86.95)	(30.4)	(82.6)	(91.3)	(100)	(100)	(69.56)	(HS)	
Ear	13	11	6	9	11	13	13	7	19.93	0.003
		(84.61)	(46.1)	(69.2)	(84.61)	(100)	(100)	(53.84)	(HS)	
Uti	11	9	1	6	8	11	11	7	30.38	0
		(81.81)	(9.09)	(54.5)	(72.72)	(100)	(100)	(63.63)	(HS)	
Burn	5	3	0	1	3	5	5	4	18.33	0.005
		(60)	(0)	(20)	(60)	(100)	(100)	(80)	(HS)	
Respiratory tract	2	0	0	0	1	2	2	1	12	0.062
		(0)	(0)	(0)	(50)	(100)	(100)	(50)	(NS)	
Total	54	43	14	36	44	54	54	35	109.3	0
		(79.62)	(25.9)	(66.6)	(81.48)	(100)	(100)	(64.81)	(HS)	
X^2		9.99	7.08	11.9	4.95	0	0	1.61		
		(S)	(NS)	(S)	(NS)	(NS)	(NS)	(NS)		
***p*- value**		0.04	0.131	0.018	0.292	1	1	0.806		

NS: Non-significant (p>0.05), S: Significant (p<0.05), HS: Highly significant difference (p<0.01).

Discussion

The study findings are in disagreement with some of the earlier findings[14] and in agreement with others.[15]

The deadliest kind of trauma are severe burns that require prompt medical attention from a specialist. The local wound microenvironment and the immunosuppressive state brought on by burn trauma are conducive to bacterial colonisation and growth.[16] The gram-negative bacterium P. aeruginosa has the highest prevalence and typically predominates in established infections among burn wound pathogens.[17] The third most common pathogen associated with catheter-related UTIs in hospitals is *P. aeruginosa*.[18] These factors have been connected to the pathogenesis of *P. aeruginosa* which results in the causes of diseases, such as otitis, burn and wound infections, RTIs and UTIs[19]

In the current study, 100 % *P. aeruginosa* isolates were positive for toxA, which is in agreement with a study.[20]A study done in Baghdad[21] reported a prevalence rate of 72%. The mexR gene was also detected in 100 % isolates in the current study, which was in line with literature.[22] The current findings showed that some clinical specimens had a greater relative frequency of virulence genes. It has been hypothesised that the location of the infection and the duration of the illness can result in the development of some virulence determinants, which in turn affects how virulent *P. aeruginosa* clinical isolates are. For instance, specific anatomical locations can increase in toxin A production and mexR expression.[23] The detection of other genes in the current study was comparable to some earlier studies,[18] but other results have also been reported.[24-27]

Differences in the distribution of virulence genes in the world may be owing to that fact that some strains of P. aeruginosa are characterised by their ability to adapt to different environments during infections, while taking advantage of the conditions of that particular site.[28] Furthermore, this may also be because strains isolated from different patients have different clinical and physiological conditions.[29]

Conclusion

Wound swabs were found to be the most common site for *pseudomonas aeruginosa*, followed by ear swabs and urine samples.

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