

Molecular Characterization of *Pseudomonas aeruginosa* isolated from Children Suffering from Urinary Tract Infection

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Abstract

A urinary tract infection (UTI) is an infection caused by bacteria that affects the bladder and nearby tissues. These are people who have no concomitant conditions, such as diabetes, an immunocompromised state, or pregnancy, and no anatomical deformity. Lower UTI or cystitis are other names for uncomplicated UTI. Bacteriuria alone does not signify a UTI in the absence of symptoms. Urinary frequency, urgency, suprapubic pain, and dysuria are typical symptoms. This study aimed to detect the percentage of *P. aeruginosa* infection in UTI patients under age 10 years, by molecular method using different genes. In this study urine samples have been collected from 100 children, 50 of those subjects cases suffering from UTI and the other 50 cases are healthy children. Bacterial DNA has extracted by all the samples and then three genes responsible for the virulence factors of *P. aeruginosa* and one reference gene (16sRNA) real time- PCR has been done to detect the presence of the bacteria. The results of 16sRNA gene showed 28 samples were positives. Las B gene showed 24 samples were positive. ExoS gene showed 11 samples positive. The gene *plcH* showed 14 samples. The results also showed that using antibiotic has a significant effect on the *plcH* gene since higher number of samples showed the presence of the gene.

Keywords; 16sRNA, Bacteriuria, ExoS, Las B, *plcH*, UTI

Introduction

An opportunistic human bacteria called Infections with Pseudomonas aeruginosa can be extremely serious, both acute and chronic. people with weakened immune systems. Pseudomonas aeruginosa is a Gram-negative rod that may infect and harm humans with impaired immune systems. The proper treatment of infections caused by P. aeruginosa bacteria is one of the biggest concerns for doctors because of the rising antibiotic resistance in the healthcare environment.(Pena, Suarez et al. 2009). Gram-negative rod infections have become a significant and fatal problem, accounting for 51,000 hospital infections annually in the USA due to severe treatment choices restrictions brought on by antimicrobial resistance.(Fujii, Seki et al. 2014). ICU stay and urinary tract infection were the two main risk factors for MBL infections, according to the multivariate analysis. MDR, or multi-drug resistance, has become widely

recognized as a serious public health concern in recent years. issue on a global scale. Additionally, MBLs strains were linked to an earlier infection start and an earlier mortality.(Bassetti, Vena et al. 2018). *Due to its vast genome, the pathogen P. aeruginosa has the potential to acquire a wide range of antibiotic resistance-related traits that affect practically all classes of drugs.* (Bassetti, Vena et al. 2018). Urinary tract infections are the most typical bacterial disease in children. patient children may experience urinary tract infections as the first sign of congenital kidney and urinary tract (CAKUT) defects or as a result of bladder dysfunction.(Oliveira and Mak 2020). Both the prevalence and severity of urinary tract infections may be influenced by bacterial virulence factors and innate human defense systems. Children's urinary tract infections show clinically in a variety of ways, and their symptoms can be difficult to diagnose.(Oliveira and Mak 2020). Healthcare-associated infections, recent urine catheterization, persistent urinary catheters, and recent urinary tract instrumentation are more common in patients with P. aeruginosa urinary tract infections (UTIs). hypertension, cognitive impairment, and/or diabetes mellitus. The mortality rate is greater and has been known to reach 20%. A poorer prognosis is linked to advanced chronic illness and insufficient decisive antimicrobial therapy. Elimination of the predisposing condition is the standard of care, along with a single antibiotic regimen that is often regarded as sufficient for treatment in the absence of septic shock. (Lamas Ferreiro, Álvarez Otero et al. 2017). P. Numerous virulence factors Pseudomonas aeruginosa contains make it easier to invade and colonize host cells. (Al-Rubaye, Yildiztugay et al. 2020). The lasB gene, which encodes the enzyme lasB elastase, is one of the genes that P. aeruginosa's virulence factors are responsible for. This enzyme actively participates in the processes of protein breakdown and necrosis, and it is also a highly toxic substance that damages tissues and invades them. (Cathcart, Quinn et al. 2011). *Additionally, P. aeruginosa carries the plcH gene, which encodes for the phospholipase enzyme. This enzyme is crucial for aiding bacteria in the host's immune evasion strategy and is a key component of pathogens invading cells and altering the phospholipid content in host cell membranes.* (Bandana, Jashandeep et al. 2018).

Methods

This study included 50 children under age 10 years that have been diagnosed with UTI attending to the urology department of the Al-Yarmook Teaching Hospital/ Baghdad, Iraq (42% of male and 58% female). Urine samples (1ml) have been collected from those subjects after requesting one of the parents. The study was approved by the medical ethical committee of the Al-

Yarmook Teaching Hospital and was in accordance with the Helsinki Declaration of 1975. The urine samples have been centrifuged then 200µl has taken from each samples and then subjected to DNA extraction. All the subjects in this study have been asked to full a questioner form by their parents.

DNA has been extracted by using Quick-DNA Fungal/Bacterial Miniprep Kit (ZYMO, USA). The eluted DNA then stored in the deep freeze till the day of PCR.

Amplification of specific region within the *P. aeruginosa* genome have been done by using specific primers. The sequences of primers pair are listed in table (1). The primers were lyophilized, then they were dissolved in free ddH₂O to give a final concentration of 100 pmol/l as stock solution. They were then kept in a stock at -20 to prepare 10 pmol/l concentration as work primer suspended, and 10 l of the stock solution in 90 l of the free ddH₂O water to reach a final volume of 100.

Table (1); Sequences of primers pair

Gene	Nucleotides sequences	Reference
LasB	GGAATGAACGAGGCGTTC TC	Strateva et al., (2008)
	GGTCCAGTAGTAGCGGTTGG	
ExoS	CTTGAAGGGACTCGACAAGG	Strateva et al., (2008)
	TTCAGGTCCGCGTAGTGAAT	
plcH	GAAGCCATGGGCTACTTCAA	Strateva et al., (2008)
	AGAGTGACGAGGAGC GGTA	
16sRNA	GGGGGATCTTCGGACCTCA	Spilker et al., (2004)
	TCCTTAGAGTGCCACCCG	

The Real time- PCR reaction mixture were prepared for each sample in a new sterile PCR tube by adding 10 µl of KAPA SYBR® FAST master mix, 5 µl of DNA, 0.5 µl of forward primer and 0.5 µl of reverse primer, the mixture then completed with 4 µl of nuclease free water. The tubes then sealed and placed onto the thermal cycler instrument, which has been programmed as follow; denaturation at 94°C for 5min then 35 cycles first at 94°C for 35s, then at 60°C for 1min. then the final step is 72 C for 7min.

The detection of the real time- PCR have been done by FAM channel during the amplification steps.

Statistical analysis

The statistical analysis of this study have been done by SPSS version 24.2 and Microsoft excel has also used. Chi-square has used for two categorical parameters. P- Value has been calculated to the probability equal to 0.05.

Results

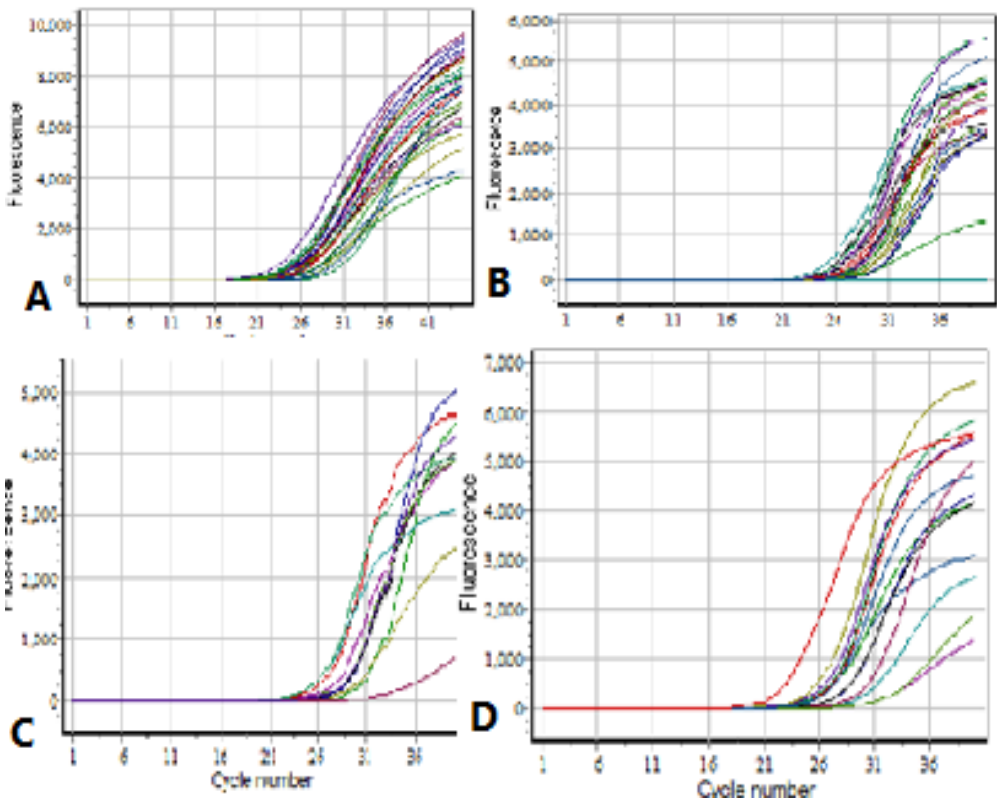
The information that related to this study have obtained from the samples. The results in table (2) summarize the percentage of the samples according to the age, gender and antibiotic receiving status. The age has been classified into age groups, the higher frequency were shown by the age group 6-10 years (44%), followed by the 3-6 years group (36%). Then the younger subjects showed the lower frequency (20%). 58% of the subjects were taking antibiotic and 42% weren't receiving any antibiotic.

Table (2); demographic informations of the studied subjects

Parameter		Frequency	%
Age	<3	10	20.0
	3-6	18	36.0
	6-10	22	44.0
Gender	male	21	42.0
	female	29	58.0
Antibiotic	Receiving	29	58.0
	None- Receiving	21	42.0

After the run of real time-PCR has been completed, the results appeared as logarithmic curves represent the amplification of the desired sequence of the genome. The results shown in figure (1). The 16sRNA gene showed 28 samples were positives. Las B gene showed 24 samples were positive. ExoS gene showed 11 samples positive. The gene plcH showed 14 samples. The sample which showed an increment in the curve shape of the real time- PCR considered positive.

Figure (1); A; amplification curves of the gene 16sRNA, B;



amplification curves of the gene LasB, C; amplification curves of the gene exoS and D; amplification curves of the gene plcH

In order to revealed the relation between the age group and resistance to bacterial infection, a comparison that shown in table (3) represent the relation between the age and the positivity of genes. The results showed a none significant relationship between the four genes and age groups.

Table (3); relation between the age groups and the positivity results of each gene

		16sRNA		Total	Chi-square	P-Value
		positive	negative			
age	<3	4	6	10	2.67	0.269
	3-6	9	9	18		
	6-10	15	7	22		
		lasB		Total	Chi-square	P-Value
		positive	negative			
age	<3	4	6	10	3.967	0.138
	3-6	6	12	18		

	6-10	14	8	22		
		exoS		Total	Chi-square	P-Value
		positive	negative			
age	<3	0	10	10	4.09	0.132
	3-6	4	14	18		
	6-10	7	15	22		
		plcH		Total	Chi-square	P-Value
		positive	negative			
age	<3	1	9	10	3.72	0.155
	3-6	4	14	18		
	6-10	9	13	22		

The effect of gender on the possession of the risk factors genes of *P. aeruginosa* are shown in the table (4). The results showed none of the genes showed a significant difference between the male and female.

Table (4); relation between the gender and the positivity results of each gene

		16sRNA		Chi-square	P-Value
		positive	negative		
gender	male	11	10	0.192	0.440
	female	17	12		
		lasB		Chi-square	P-Value
		positive	negative		
gender	male	10	11	0.002	0.598
	female	14	15		
		exoS		Chi-square	P-Value
		positive	negative		
gender	male	4	17	0.184	0.471
	female	7	22		
		plcH		Chi-square	P-Value
		positive	negative		
gender	male	5	16	0.315	0.407
	female	9	20		

The effect of using antibiotic have showed a significant effect on both the presence of the infection and in the presence of the genes. The number of negative samples was higher in the group of subjects that were using the antibiotic and this difference was significant (0.03). The using of antibiotic has a

significant effect on the *pcl H* gene since higher number of samples showed the presence of the gene (220 compare to patients that not receiving antibiotic(2).

Table (5); relation between the receiving of antibiotic and the positivity results of each gene

		16sRNA		Chi-square	P-Value
		positive	negative		
antibiotic	receiving	7	22	4.723	0.030
	none	8	13		
		lasB		Chi-square	P-Value
		positive	negative		
antibiotic	receiving	17	12	3.12	0.062
	none	7	14		
		exoS		Chi-square	P-Value
		positive	negative		
antibiotic	receiving	8	21	1.256	0.221
	none	3	18		
		plcH		Chi-square	P-Value
		positive	negative		
antibiotic	receiving	22	7	6.131	0.013
	none	2	19		

Discussion

due to its inherent tolerance to several antimicrobial treatments, a nosocomial infection. According to a survey conducted in the United States, *P. aeruginosa* is the second-leading cause of nosocomial pneumonia, the seventh-most common cause of bloodstream infections, and the third-most common cause of urinary tract infections, all of which occur often. (14 percent -16 percent). (Poole and Hazlett 2011). A recent study discovered that the frequency of isolated bacterial species increases with urine pH and age, particularly in children under the age of one year old, and when urine pH approaches 8. The combination of a number of variables, including the pathogenic characteristics of the pathogen The urine pH in a UTI is determined by the host's immunological and physiological reactions. The current study reveals that *P. mirabilis* or *P. aeruginosa* displayed the least acidic pH, which is consistent with the relationship between bacterial species and urine pH. As a result, an age-related pathogen pattern can be identified due to the acidic urine in infants and the more alkaline urine in older children. Most people think that *P. aeruginosa* and *P. mirabilis* are bacteria that can create urease as the etiological process of

disease.(Mobley and Hausinger 1989). Urease converts the urea in urine urine's pH is increased as a result of the conversion of CO₂ and ammonia. Some causative uropathogens are related to age and some causative uropathogens are related to age and urine pH in children. Particularly with *P. mirabilis* or *P. aeruginosa* infections, less acidic urine is linked. *E. coli*, on the other hand, seldom makes urease and cannot alkalize the urine.(Mobley and Hausinger 1989). *P. aeruginosa* is still quite vulnerable exhibits some susceptibility to cephalosporins, fluoroquinolones, and colistin. despite a recent research showing it was extremely resistant to carbapenems and several other medications.(Hu, Cao et al. 2019). Non-*E. coli* uropathogens were more prevalent in men than in females, as demonstrated in a prior research. Expanding the use of empiric antibiotics in males requires more research. (Hameed, Al Nafeesah et al. 2019). In addition to the traditional culture, uropathogen identification utilizing 16S rRNA analysis may be used to treat febrile UTI in young infants. *P. aeruginosa* is capable of directly generate and release virulence agents *Pseudomonas aeruginosa* (PA), an opportunistic bacterium, is the main source of pervasive and overwhelming hospital acquired infections due to a plethora of virulence factors, including toxins. Therefore, in clinical settings, these traits are the main factor contributing to an increase in morbidity and duration of hospital stay. (Yousefi-Avarvand, Khashei et al. 2015). Two major classes of enzymes and poisons are presented as the secretory virulence factors for *P. aeruginosa*. The enzymes *exoY*, *exoS*, *exoT*, and *exoU* have been designated as Exoenzymes. *ExoS* and *ExoT*, two ADP-ribosylating enzymes, *ExoU*, an acute cytolytic factor, and *ExoY*, an adenylate cyclase, are powerful exotoxins that this secretion system introduces via a cell contact-mediated method into the cytoplasm of the host cells. One of *P. aeruginosa*'s most crucial virulence factors, *ExoU*, is connected to a higher risk of early clinical death. (Kaszab, Szoboszlay et al. 2011). *ExoS* and *exoT* are bifunctional toxins that have the ability to activate both GTPases and ADP-ribosyltransferases. Compared to *exoS*, *exoT* has less ADP-ribosyltransferase activity. (Wareham and Curtis 2007). Both in vitro and in vivo tests have showed that *exoS* and *exoU* were the main cytotoxins. *ExoS* prevalence was previously investigated in relation to the presence of carbapenemase genes. To ascertain the relationship between *exoS* gene prevalence and carbapenemase production, more research is required.(Park and Koo 2022). This may be due to the cytotoxin genes *exoS* and *exoU* being present in *P. aeruginosa* as variable features based on the illness's location or history, and their existence. (Azimi, Kafil et al. 2016). The fact that these isolates come from various clinical locations and were obtained at various stages of infection may be the cause of the variation in virulence factors produced by clinical isolates.

Different virulence genes are distributed differently in clinical and environmental isolates., indicating that this pathogen has evolved to adapt to various environments.(Heidary, Bandani et al. 2016). Hemolytic phospholipase C (PlcH) hydrolyzes a number of phospholipids found in the host cell membrane, including the sphingomyelin and phosphatidylcholine that are connected with the host. The ability to considerably injure tissue has been connected to high levels of hemolytic activity, and PlcH, a hemolytic protein, is in charge of pro-inflammatory activity and the inhibition of neutrophil respiratory burst activity. Additionally, LasB elastase breaks down cellular structural proteins like collagen. Elastin, Non-collagen proteins and the enzyme LasA promote the elastolytic activity of LasB. the most gene is present in all clinical isolates, highlighting its significance to *P. aeruginosa* survival in various conditions. The previous study found that bacteria with the *lasB* gene are similar to those with the *plcH* gene, increasing the likelihood that these isolates will be pathogenic. compared to those containing only one of the two genes. (Hasan, Hussein et al. 2021). *P. aeruginosa* isolated from Egyptian human clinical samples was shown to possess both features of antibiotic resistance and pathogenicity. in reports. Our findings showed that human infections frequently contain all of the *tox A*, *lasB*, *plcH*, and *plcN* genes.(Naga, Abdulrazzaq et al. 2018).

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