Original Article

Comparative Analysis of Four Genetic Markers for *Pseudomonas aeruginosa* Detection in Pediatric UTIs: Influence of Antibiotic Exposure on Gene Prevalence

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Abstract

Background: A urinary tract infection (UTI) is a health condition that affects the bladder and adjacent tissues and is brought on by bacteria. **Objective:** This study aimed to detect the percentage of *Pseudomonas aeruginosa* infection in UTI patients under the age of 10 years using molecular methods with different genes. **Materials and Methods:** In this study, urine samples have been collected from 100 children, 50 of whom have UTI, while the other 50 are healthy children. Bacterial DNA was extracted from all the samples, followed by real-time polymerase chain reaction (PCR) using three genes responsible for the virulence factors of *P. aeruginosa* and one reference gene (16S rRNA) to detect the presence of the bacteria. **Results:** The results of 16S rRNA gene showed that 28 samples were positive. LasB gene showed that 24 samples were positive. ExoS gene showed that 11 samples were positive. plcH gene showed that 14 samples were positive. Additionally, the findings show that the use of antibiotics has a significant impact on the presence of pclH gene, as a higher number of samples showed the gene's presence. The variations of *P. aeruginosa* detection highlight the significance of choosing the appropriate molecular marker for diagnostic accuracy. The 16S rRNA gene detected the highest number of positive samples. **Conclusions:** The positive correlation between antibiotic usage and the detection rate of the plcH gene is observed. The result of this study raises the possibility of a connection between the existence of the plcH gene in *P. aeruginosa* and antibiotic resistance.

Keywords: 16S rRNA gene, bacteriuria, ExoS, LasB, pclH, UTI

Introduction

An opportunistic human bacteria called infections with *Pseudomonas aeruginosa* can be extremely serious, both acute and chronic, especially for people with weakened immune systems. "*P. 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 health care environment.[1] Due to severe limitations in treatment options brought on by antibiotic resistance, Gram-negative rod infections—which cause 51,000 hospital infections yearly in the USA—have grown to be a serious and lethal concern.[2] Intensive care unit (ICU) stay and urinary tract infection (UTI) were identified as the two main risk factors for metallo-β-lactamase (MBL) infections, according to the multivariate analysis. Multidrug resistance has become widely recognized as a serious public health issue on a global scale in recent years. Additionally, MBLs strains were linked to an earlier infection start and an earlier mortality. 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.[3] UTIs are the most typical bacterial

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disease in children. Pediatric patients may experience UTIs as the first sign of congenital kidney and urinary tract defects or as a result of bladder dysfunction. Both the prevalence and severity of UTIs may be influenced by bacterial virulence factors and innate human defense systems. Children's UTIs show clinically in a variety of ways, and their symptoms can be difficult to diagnose.[4] Health care-associated infections, recent urine catheterization, persistent urinary catheters, and recent urinary tract instrumentation are more common in patients with *P. aeruginosa* UTIs than in those without. Patients with UTIs may also have comorbidities such as 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.[5] *P. aeruginosa* contains numerous virulence factors that make it easier to invade and colonize host cells.[6] The LasB gene, which encodes the enzyme LasB elastase, is one of the genes responsible for *P. aeruginosa* virulence factors. It orchestrates several physiopathological events during bacteria-host interactions. 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.[7] 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.[8]

Materials and Methods

This study included 50 children under the age of 10 years who had been diagnosed with UTI and were attending the Urology Department of the Al-Yarmook Teaching Hospital in Baghdad, Iraq (42% of male and 58% female). Urine samples (1 mL) have been collected from those subjects after requesting one of the parents. The urine samples were centrifuged, and then 200 µL were taken from each sample. To confirm the presence of *P. aeruginosa* in the samples, a dual approach was employed using the VITEK® system and a targeted selective medium. Cetrimide Agar, a selective medium, was utilized for its specificity in promoting the growth of *P. aeruginosa*. This medium inhibits the growth of other bacterial species, facilitating the accurate identification of *P. aeruginosa*. The combination of these sophisticated techniques provided a robust and reliable method for confirming the bacterium's presence, which is pivotal for accurate diagnostics and effective treatment strategies. Subsequently, the samples were subjected to DNA extraction. All the subjects in this study were asked to fill out a questionnaire form by their parents.

Table 1: Sequences of primers pair

Gene	Nucleotides sequences	Reference
LasB	GGAATGAACGAGGCGTTC TC GGTCCAGTAGTAGCGGTTGG	Strateva *et al.* (2008)[9]
ExoS	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCGTAGTGAAT	Strateva *et al.* (2008)[9]
plcH	GAAGCCATGGGCTACTTCAA AGAGTGACGAGGAGC GGTAG	Strateva *et al.* (2008)[9]
16S rRNA	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	Spilker *et al.* (2004)[10]

Quick-DNA Fungal/Bacterial Miniprep Kit (ZYMO, USA) was used to extract DNA. The deep freezer was then used to keep the eluted DNA until the day of the polymerase chain reaction (PCR).

Using specific primers, amplification of a certain area of the *P. aeruginosa* genome has been carried out. Table 1 includes a list of the primer pair sequences. The primers were lyophilized before being dissolved in free ddH_2O to produce a stock solution with a final concentration of 100 pmol/L. Then, "they were stored at −20°C to create a 10 pmol/L concentration as suspended working primers. For use, 10 L of the stock solution was mixed with 90 L of free ddH_2O water to make a final volume of 100."

In a new sterile PCR tube, the real-time PCR reaction mixture was created for each sample by adding 10 µL of KAPA SYBR® FAST master mix, 5 µL of DNA, 0.5 µL of forward primer, 0.5 µL of reverse primer, and 4 µL of nuclease-free water. The tubes were then sealed and placed in the thermal cycler apparatus, which had been programmed to run 35 cycles: initially at 94°C for 35 s, followed by 60°C for 1 min, with a final denaturation step at 94°C for 5 min, and then 72°C for 7 min as the last phase.

The detection of the real-time PCR was done using the FAM channel during the amplification steps.

Statistical analysis

Microsoft Excel and SPSS (IBM, United States) version 24.2 were both utilized in the statistical analysis of this study. Two categorical parameters were analyzed using the Chi-square test. The *P* value was determined to be 0.05.

Ethical approval

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.

Results

The information related to this study has been 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 was classified into age groups, with the highest frequency observed in the 6–10 years age group (44%), followed by the 3–6 years group (36%). The youngest subjects showed the lowest frequency (20%). Among the subjects, 58% were taking antibiotics, while 42% were not receiving any antibiotic.

After the run of real-time PCR has been completed, the results appeared as logarithmic curves representing the amplification of the desired sequence of the genome. The results are shown in Figure 1. The 16S rRNA gene showed that 28 samples were positive. The LasB gene showed that 24 samples were positive. The ExoS gene showed that 11 samples were positive. The plcH gene showed that 14 samples were positive. Samples that exhibited an increment in the curve shape during real-time PCR were considered positive.

Table 2: Demographic data about the participants under investigation

Parameter		Frequency	%
Age	<3	10	20.0
	3–6	18	36.0
	6–10	22	44.0
Gender	M	21	42.0
	F	29	58.0
Antibiotic	Receiving	29	58.0
	None	21	42.0

To reveal the relationship between age groups and resistance to bacterial infection, a comparison shown in Table 3 represents the relationship between the age and the positivity of genes. According to the results of this study, there is no significant relationship between the four genes and age groups.

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

The effect of using antibiotics has shown a significant effect on both the presence of the infection and 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 use of antibiotics has a significant effect on the pclH gene since higher number of samples showed the presence of the gene (220 compared to patients not receiving antibiotics [Table 5].[2]

Discussion

P. aeruginosa, due to its inherent tolerance to several antimicrobial treatments, is a significant cause of nosocomial infections. It ranks as the second most

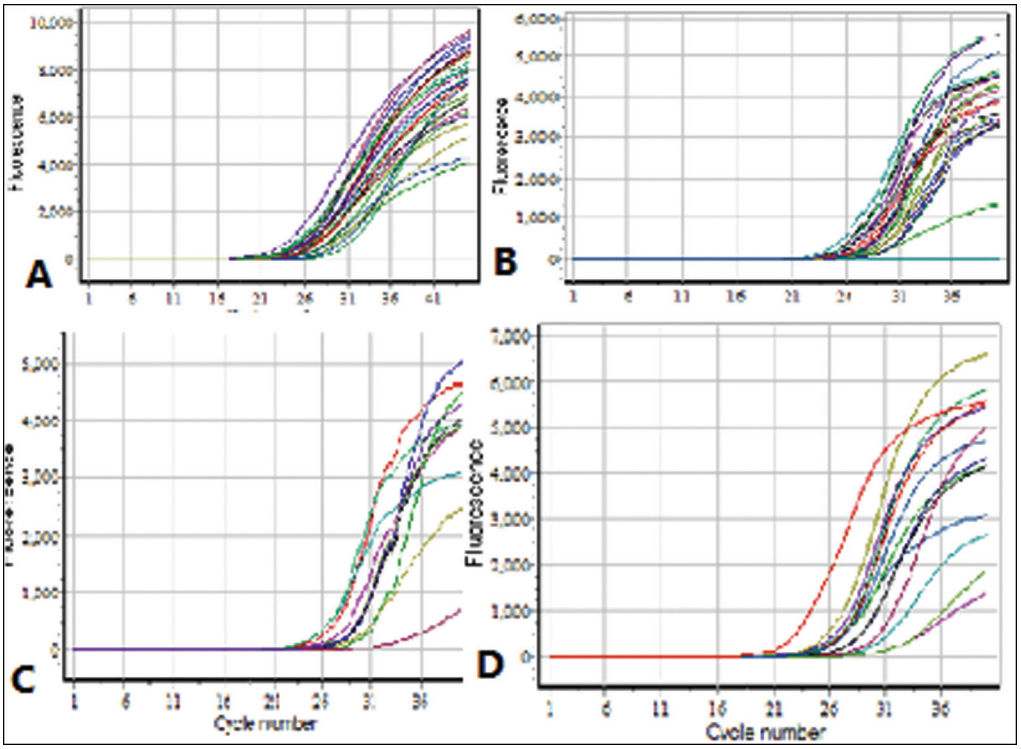

Figure 1: Amplification curves of the genes (A) 16S rRNA, (B) LasB, (C) ExoS, and (D) plcH

Table 3: Relation between the age groups and the positivity results of each gene

		Positive	Negative	Total	Chi-square	*P* value
16S rRNA						
Age	<3	4	6	10	2.67	0.269
	3–6	9	9	18		
	6–10	15	7	22		
LasB						
Age	<3	4	6	10	3.967	0.138
	3–6	6	12	18		
	6–10	14	8	22		
ExoS						
Age	<3	0	10	10	4.09	0.132
	3–6	4	14	18		
	6–10	7	15	22		
plcH						
Age	<3	1	9	10	3.72	0.155
	3–6	4	14	18		
	6–10	9	13	22		

Table 4: Relation between the gender and the positivity results of each gene

Gender	Positive	Negative	Chi-square	*P* value
16S rRNA				
M	11	10	0.192	0.440
F	17	12		
LasB				
M	10	11	0.002	0.598
F	14	15		
ExoS				
M	4	17	0.184	0.471
F	7	22		
plcH				
M	5	16	0.315	0.407
F	9	20		

common cause of nosocomial pneumonia, the seventh most common source of bloodstream infections, and the third most common cause of UTIs, with occurrence rates ranging from 14% to 16%, according to a survey conducted in the United States.[11] A recent study discovered that the frequency of isolated bacterial species increases with urine pH and age, especially in children under the age of 1 year old, particularly 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 *Proteus mirabilis* or *P. aeruginosa* displayed the least acidic pH, which is consistent with the relationship between bacterial species and urine pH. Due to newborn acidic urine and older children's more alkaline urine, an age-related pathogen pattern can be seen. Most people think that *P. aeruginosa* and *P. mirabilis* are bacteria that can create urease as the etiological process of disease.[12] Urease converts urea in urine, resulting in an increase in urine pH due to the conversion of CO_2 and ammonia. Some causative uropathogens in children are related to age, while others are related to urine pH. Infections, particularly with *P. mirabilis* or *P. aeruginosa*, are associated with less acidic urine. *Escherichia coli*, on the other hand, seldom makes urease and cannot alkalinize the urine.[12] While *P. aeruginosa* exhibits some susceptibility to cephalosporins, fluoroquinolones, and colistin, recent research has shown it to be extremely resistant to carbapenems and several other medications.[13]

Non-*E. coli* uropathogens were found to be more prevalent in men than in women, as demonstrated in previous research. Expanding the use of empiric antibiotics in males requires more research.[14] In addition to the traditional culture, uropathogen identification utilizing 16S rRNA analysis may be used to treat febrile UTI in young infants. "*P. aeruginosa*, an opportunistic bacterium, is capable of directly generating and releasing virulence agents. It 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.[15] 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 adenosine diphosphate (ADP)-ribosylatin enzymes, ExoU, an acute cytolytic factor, and ExoY, an adenilate cyclase, are powerful exotoxins introduced into the cytoplasm of the host cells via a cell contact-mediated method by this secretion system. One of *P. aeruginosa* most crucial virulence factors, ExoU, is connected to

Table 5: Relation between the receiving of antibiotic and the positivity results of each gene

		Positive	Negative	Chi-square	*P* value
16S rRNA					
Antibiotic	Receiving	7	22	4.723	0.030
	None	8	13		
LasB					
Antibiotic	Receiving	17	12	3.12	0.062
	None	7	14		
ExoS					
Antibiotic	Receiving	8	12	1.256	0.221
	None	3	18		
plcH					
Antibiotic	Receiving	22	7	6.131	0.013
	None	2	19		

a higher risk of early clinical death.[16] ExoS and ExoT are bifunctional toxins with the ability to activate both GTPases and ADP-ribosyltransferases. Compared to ExoS, ExoT has less ADP-ribosyltransferase activity.[17] Both *in vitro* and *in vivo* tests have shown 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.[18] This variability may be attributed to the presence of cytotoxin genes, such as ExoS and ExoU, in *P. aeruginosa*, which can vary based on the location or history of the illness.[19] 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 in clinical and environmental isolates, indicating that this pathogen has evolved to adapt to various environments.[20] Hemolytic phospholipase C (plcH) hydrolyzes a number of phospholipids found in the host cell membrane, including the sphingomyelin and phosphatidylcholine, which 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 such as 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."[21] *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 toxA, LasB, plcH, and plcN genes.[22]

Conclusions

The variations of *P. aeruginosa* detection highlight the significance of choosing the appropriate molecular marker for diagnostic accuracy. The 16S rRNA gene detected the highest number of positive samples. The positive correlation between antibiotic usage and the detection rate of the plcH gene is observed. The result of this study raises the possibility of a connection between the existence of the plcH gene in *P. aeruginosa* and antibiotic resistance.

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Conflicts of interest

There are no conflicts of interest.

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