Molecular identification, prevalence, and antibiotic resistance of Pseudomonas aeruginosa isolated from clinical and medical waste samples in Baghdad City, Iraq

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Abstract

Identification of *P. aeruginosa*, determining the prevalence of this pathogen among clinical and medical waste samples, and studying the antibiogram profile of isolates were the objectives of this study. A total of 394 (300 clinical & 94 medical waste) samples were collected during February 2020- October 2020. Morphological characterization, biochemical behavior, and VITEK 2 system identified 14 (5%) and 6 (8.3%) of the presumptive clinical and medical waste isolates respectively as *P. aeruginosa*. 16S rRNA and *gyrB* genes confirmed that 8 (2.9%) and 4 (5.6%) clinical and medical waste isolates respectively were *P. aeruginosa*. Statistically, the difference between the prevalence of *P. aeruginosa* among clinical and that among medical waste samples was non-significant (P≤0.05). The percentages of resistance showed by *P. aeruginosa* strains were 91.7% for Ticarciliin; 75% Ticarcillin/Clavulanic acid; 66.7% Ceftazidime, Imipenem, & Ciprofloxacin; 58.3% Gentamicin & Tobramycin; 50% Meropenem; 41.7% Ticarcillin/Clavulanic acid; 33.3% Amikacin, 16.6% Cefepime; and 8.3% for Colistin. In conclusion, medical wastes are a potential source of infection caused by *P. aeruginosa*, treatment of nosocomial infections should be guided by antibiotic susceptibility testing, and colistin was the most effective antibiotic.

Keywords: Pseudomonas aeruginosa, prevalence, antibiotic resistance, clinical samples, medical waste samples

Introduction

P. aeruginosa is an opportunistic pathogen with acute to chronic infections in humans. It often infects people with low immunity and those infections lead to a high fatality rate in those needing mechanically assisted ventilation or burn patients. This pathogen also plays a substantial role in chronic respiratory infection, especially for those with cystic fibrosis (CF) and several other chronic respiratory system infections[1,2].

Treatment of this pathogen can be difficult because of its natural and acquired resistance to antibiotics. It has a remarkable ability to acquire antibiotic-resistance genes, to spread from patient to patient, and to persist in the hospital environment[3]. It's the third most common cause of nosocomial infections after *Staphylococcus aureus* and *Escherichia coli*[4].

Medical wastes include wastes generated by health care activities and produced by medical institutions (private or public), medical research facilities, or laboratories[5]. They are classified into four categories: hazardous; radioactive; pharmaceutical; and Infectious wastes which include gloves, bandages, swabs, cultures, any waste contaminated with blood and body fluids, *etc* [6,7].

The contagious risk posed by medical wastes to the environment and human health is the possibility of presence of pathogenic microorganisms. In many developing countries, medical wastes are handled and disposed together with non-medical wastes, which is creating a vital and even fatal health risk to hospitalized patients, health care workers and the general public[7]. A recent local study found that there were ineffective, insufficient, and incorrect isolation, collecting, storing, treatment, and safe disposal of medical wastes in the sampled public hospitals of Baghdad[8].

Materials and Methods

Samples collection

A total of 394 (300 medical and 94 clinical waste) samples were collected during February 2020-October 2020 from hospitals in Baghdad, Iraq.

Culture and bacterial identification

By using sterile cotton swabs, samples were collected and then transported to laboratory immediately. The swabs were streaked on nutrient, MacConkey, blood, and on the selective medium *Pseudomonas* cetrimide agar (PCA) plates and then they were kept overnight at 37 °C to observe colony morphology. Colonies that are suspected as *P. aeruginosa*, were processed further for conventional biochemical tests: indole, cytochrome oxidase, catalase, motility, and citrate utilization tests. VITEK

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2 system and molecular markers based on the amplification of 16S rRNA and *gyrB* genes were used to characterize and confirm the identification of presumptive *P. aeruginosa* isolates.

DNA extraction

From bacterial growth, genomic DNA was isolated according to the protocol of Wizard® Genomic DNA Purification Kit[9]. In order to detect the concentration of extracted DNA, Quantus Fluorometer was used. This step was done in order to detect the goodness of samples for downstream applications. One microliter of DNA and one hundred and ninety-nine microliter of diluted Quanty Flour Dye was mixed. After five minutes incubation at room temperature, DNA concentration values were detected.

Polymerase chain reaction (PCR) cycle

The DNA of isolates was targeted for 16S rRNA and *gyrB* genes using primers listed in Table 1. A reaction mixture (20 µl) contained 10 µl of Master Mix, 5 µl of nuclease free water, 3 µl of DNA, and 1 µl of each primer. The experiment was continued according to the following program: initial denaturation at 95°C for 5 min, 30 cycles at 95°C for 30 Sec, 55/58°C for 30 Sec, 72°C for 30 Sec. and then a final extension at 72°C for 7 min as shown in Table 2. The analysis of PCR products were done using 1.5% agarose gel electrophoresis and by using gel imaging system, the ethidium bromide stained bands in gel were visualized.

Table 1: Primer sequences used for PCR.

Genes	Primer sequence (5'-3')	Product size (bp)	Annealing Temp. (°C)	References
16S rRNA-F 16S rRNA-R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	58	10
gyrB-F *gyrB*-R	AAGTACGAAGGCGGTCTGAA GTTGTTGGTGAAGCAGAGCA	171	55	11

Table 2: PCR program

Steps	°C	m:s	Cycle
Initial denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	55/58	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Antibiotic susceptibility test

Susceptibility to antibiotics was determined by Vitek 2 system. Every 15 min for 18 hrs, this apparatus automatically measures a turbidity signal for each test well that contains an antibiotic. Growth curves are generated by using these data and by comparing with a control, the minimum inhibitory concentration (MIC) of each antibiotic is estimated[12]. MIC results (µg/ml) were translated into clinical categories (Resistant, Intermediate, and Susceptible) by comparing with Breakpoints for susceptibility category determination recommended by the clinical and laboratory standards institute (CLSI) guidelines.

Statistical Analysis

The effect of difference factors in study parameters was detected using the Statistical Analysis System-SAS (2012) program. For significant compare between percentages in this study, Chi-square test was used [13].

Results and discussion

Identification of P. aeruginosa

Morphological characterization and biochemical behavior identified 14 (5%) and 6 (8.3%) of the presumptive clinical and medical waste isolates respectively as *P. aeruginosa*. These 20 isolates were able to grow on cetrimide agar as a selective medium and appeared as smooth and greenish-yellow colony as well as it appeared as lactose-non-fermenting mucoid colonies on MacConkey agar; beta-hemolytic brownish gray colonies on blood agar; and smooth greenish blue colonies on nutrient agar with grape - like odor. They showed negative result for indole test and positive results for cytochrome oxidase, catalase, mannitol motility, and citrate utilization tests. These results agreed with what Bhuiya *et al.*, ; Quinn *et al.*, ; Pitt and Simpson ; and Carter and Wise stated for identifying *P. aeruginosa* [14-17].

Although the morphological characterization and biochemical tests used to identify bacteria appear to be more strong than previously, it still lacks to rapidity, ease, and reliability. So, the use of automated systems in order to solve the challenges of rapid and accurate identification is necessary. Confirmatory identification with the VITEK 2 system was performed with plastic cards referred to as Gram-negative card BioMérieux, according to the manufacturer's instructions. The VITEK 2 system correctly identified 20 0f 20 (100%) isolates of *P. aeruginosa*.

16S rRNA and *gyrB* genes confirmed that 8 (2.9%) and 4 (5.6%) clinical and medical waste isolates respectively were *P. aeruginosa* (figs. 1 and 2)

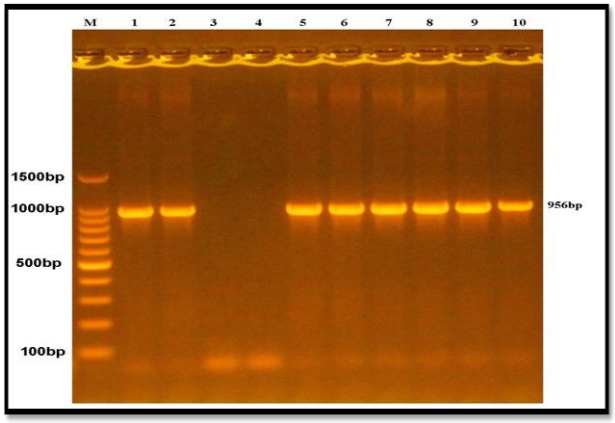

Figure 1: Results of the amplification of 16SrRNA gene of Pseudomonas aeruginosa samples

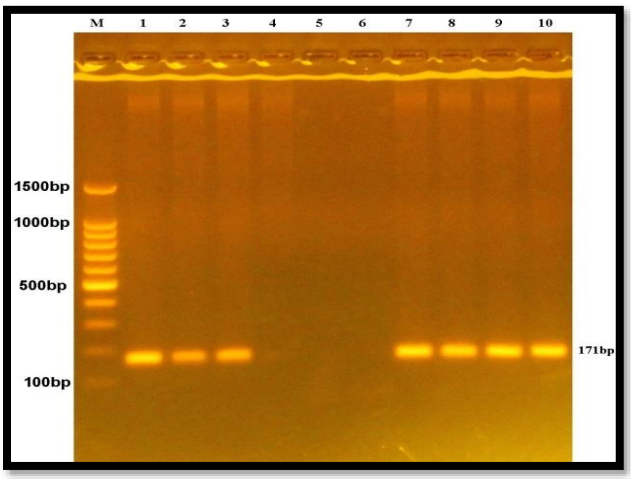

Figure 2: Results of the amplification of gyrB gene of Pseudomonas aeruginosa samples

Because of the presence of 16S rRNA gene in all bacteria and the different evolutionary rates depending on the gene region considered, this gene has historically been used for classification of isolates[18]. However, different studies have shown that the sequences of *gyrB* gene of other species of *Pseudomonas* and *P. aeruginosa* showed a higher variance than 16S rRNA genes. The reason is that the molecular evolution rate of 16S rRNA gene is lower than that of *gyrB* gene and *gyrB* gene is rarely transmitted horizontally. Therefore, the 16S rRNA gene is a less appropriate target than *gyrB* gene for the identification of the *pseudomonas* species[19]. On the other hand, the *gyrB* primer sequences designed by Qin *et al.*, are 100% identical to the *gyrB* gene from *Pseudomonas composti* as shown by basic local alignment search tool (BLAST) results for *gyrB* primers[20]. *P. composti* is a novel species that was isolated from compost and reported by Gibello *et al.*,[21]. The *gyrB* gene fragment of this strain has a 99% identity to *P. aeruginosa*, and therefore, false positive results with *gyrB* uniplex PCR can be obtained.

The prevalence of Pseudomonas aeruginosa among samples

From 280 clinical samples which were positive for bacterial growth, 8 *P. aeruginosa* isolates were obtained with a prevalence rate of 2.9%. Similar prevalence rate of 2.1% was obtained by Okon *et al.*, [22]. In comparison, relatively higher prevalence rate of 5.1% and 9.3% were reported by Shrestha *et al.*, [23] and Srinivas *et al.*, [24] respectively. The varied prevalence of *P. aeruginosa* among several studies may attribute to the type of clinical samples, type of hospitals and geographical locations [25].

From 72 medical waste samples which were positive for bacterial growth, 4 *P. aeruginosa* isolates were obtained with a prevalence rate of 5.6%. Similar prevalence rate of 5.1% was obtained by Phoon *et al.*, [26]. While in the study of Karami *et al.*, [27], the prevalence of *P. aeruginosa* isolated from hospital environmental samples was 18.5% which is greater than that reported in this study. Differences in geographical location and hygienic strategies can be the reason behind the difference in prevalence rate among several studies.

Statistically, the difference between the prevalence of *P. aeruginosa* among clinical and that among medical waste samples was non-significant (P≤0.05).

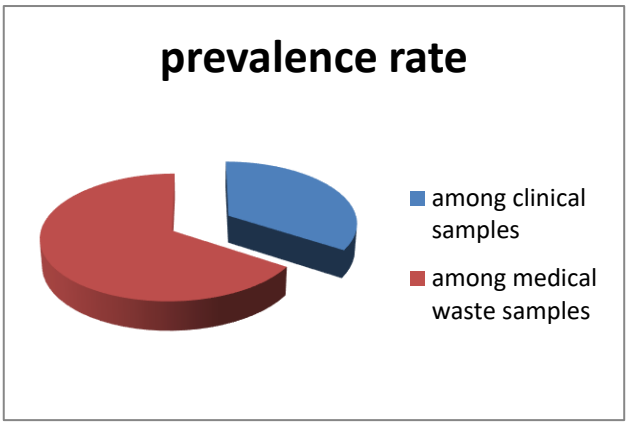

Figure 3: The prevalence of Pseudomonas aeruginosa among samples

Antibiotic susceptibility test

Among antibiotics used in this study, Colistin was the most effective antibiotic against isolates (91.7% isolates were susceptible) as shown in table (3). This finding is in agreement with previous studies: 96%; 97.5%; and 98.03% Colistin susceptible *P. aeruginosa* isolates recorded by van Burgh *et al.*, [28] in Kurdistan, Iraq; Izadi Pour Jahromi *et al.*, [29] in Iran; and Hussein *et al.*, [30] in Wasit, Iraq respectively and is in disagreement with 60% and 65% Colistin susceptible *P. aeruginosa* isolates recorded by Khudair and Mahmood[31] in Baghdad, Iraq and Najeeb and Al-Taai [32] in Baquba City, Iraq.

Table 3: Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* isolates

Antibiotics	N (%)		
	S	R	I
Ticarciliin	1 (8.3%)	11 (91.7%)	
Ticarcillin/Clavulanic acid	4 (33.3%)	5 (41.7%)	3 (25%)
Piperacillin	1 (8.3%)	9 (75%)	2 (16.6%)
Ceftazidime	2 (16.6%)	8 (66.7%)	2 (16.6%)
Cefepime	9 (75%)	2 (16.6%)	1 (8.3%)
Imipenem	3 (25%)	8 (66.7%)	1 (8.3%)
Meropenem	3 (25%)	6 (50%)	3 (25%)
Amikacin	5 (41.7%)	4 (33.3%)	3 (25%)
Gentamicin	3 (25%)	7 (58.3%)	2 (16.6%)
Tobramycin	4 (33.3%)	7 (58.3%)	1 (8.3%)
Ciprofloxacin	4 33.3%)	8 (66.7%)	
Colistin	11 (91.7%)	1 (8.3%)	

In this study, only one isolate was resistant to Colistin. This might be attributed to the less frequent use of this antibiotic because of the un sustained availability in local markets and hospitals. However, comparing with other previous studies: Biswal *et al.*,[33] in India; Ece *et al.*,[34] in Turkey; Ali *et al.*,[35] in Pakistan; Al-Delaimi and Nabeel[36] in Duhok, Iraq; and AL-Fridawy *et al.*,[37] in Baghdad, Iraq which reported 100% susceptibility to Colistin, even the only Colistin resistant isolate found in this study is very worrying. The emergence of Colistin resistance in various countries has become one of the global worrying as Colistin is considered the last recourse for the treatment of severe infections caused by multidrug- resistant (MDR) and extensively drug-resistant (XDR) strains[38].

At the present time, the spread of Colistin susceptibility is high. However, in spite of most of the studies reporting resistance rates below 10%, there are some other studies reporting low susceptibility rates. This spread may differ among geographical regions with different treatment strategies and over time. According to Nation *et al.*,[39] clinicians do not have access to Colistin in some countries, like South Africa and Japan, while they have only the parenteral formulation of Colistin in a few areas of the world, like Australia and Europe, and they can use either the polymyxin B or Colistin parenteral for treatment in the Brazil, United States, Malaysia, and Singapore.

Ceftazidime, Carbapenems, aminoglycosides, and Fluoroquinolones are important antibiotics for the treatment of *Pseudomonas* infections. So it is essential to highlight the presence of the 66.7% ceftazidime, imipenem, and ciprofloxacin resistant; 50% meropenem resistant; and 58.3% gentamicin and tobramycin resistant *P. aeruginosa* isolates in this study. The percentage of resistance to ceftazidime recorded here was similar to 66% reported by a local study carried out by Sulaiman and Abdulhasan[40]. Another local study showed lower resistance that reached to 17.5% [41]. This represents a challenge as Ceftazidime is a third generation Cephalosporin that is the most active Cephalosporin available against *P. aeruginosa*. However, resistance might be caused by the random use of Cephalosporin (third generation) as broad spectrum experimental therapy and the secretion of extended spectrum β -lactamases (ESBL) mediate the resistance by hydrolyzing β-lactam ring of β-lactams[42].

The percentage of resistance to imipenem (66.7%) recorded here was similar to 66.6% reported by a local study carried out by Al-abedi and Al-Mayahi[43]. However, comparing with local study which reported 100% imipenem and meropenem sensitive *P. aeruginosa* isolates carried out by Al-Saffar and Jarallah[44], our findings are quite alarming as carbapenems including imipenem and meropenem have been considered a last choice against many antibiotic resistant bacterial infections and are currently the choice of antibiotic for multidrug resistant *P. aeruginosa* infections. The mechanisms of resistance to carbapenems in gram-negative bacteria *P. aeruginosa* including: loss or reduced expression of the outer membrane protein (OprD) that could give a resistance to imipenem and a reduced susceptibility to meropenem; acquired Amber class B metallo-beta-lactamase (MBLs) production that give resistance to imipenem and meropenem; and overexpression activity of the efflux pumps MexAB-OprM that may give a reduced susceptibility to meropenem, but don't affect susceptibility to imipenem [45, 46].

The percentages of resistance to aminoglycosides (amikacin, gentamicin, & tobramycin) recorded here were lower than those (60.9% amikacin resistant, 73.9% gentamicin resistant, & 71% tobramycin resistant *P. aeruginosa* isolates) reported by a local study carried out by AL-Rubaye *et al.*, [47] As well as, our findings were lower than another local study findings (91% amikacin resistant, 93% gentamicin resistant, & 94% tobramycin resistant *P. aeruginosa* isolates) reported by Qader *et al.*,[48]. Aminoglycosides are integral to the treatment of *P. aeruginosa*[49].

However, resistance to these antibiotics often develops due to the upregulation or acquisition of genes that encode efflux systems or inactivating enzymes. production of inactivating enzymes is the most common resistance mechanism to aminoglycosides in gram negative bacteria[50].

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