Original Article

Pseudomonas aeruginosa detection and identification in burn and wound clinical samples from Al Muthanna hospitals in Iraq using 16S rRNA gene sequencing and measurement of antibiotic resistance

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Abstract

A Objective: This study's objective was to identify Pseudomonas aeruginosa, which was isolated from clinical samples (burn and wound) at Al Muthanna hospitals in Iraq by sequencing the 16S rRNA gene and studying its resistance to some antibiotics

Methods: Fifty-five bacterial isolates were obtained from clinical samples (burns and wounds) from Al Muthanna hospitals in Iraq, identified as P. aeruginosa using enrichment selective

media and biochemical tests. For identification of P. aeruginosa at the DNA level, DNA was extracted from P. aeruginosa using the genomic DNA extraction kit. The conventional PCR technique is carried out based on a specific primer for 16S rRNA. The amplified PCR products were sequenced by Macrogen Inc. in Seoul, South Korea. The susceptibility of P. aeruginosa isolates to 20 different antibiotics was investigated according to the (CLSI).

Results: 55 P. aeruginosa isolates produces various colours on enrichment selective media, including blue, green, and yellow-green. Sequence analysis by BLASTn displayed 10 local isolates had 96 % similarity to the detected P. aeruginosa strain recoded earlier in the GenBank the rest of the local analysis samples were also recognised as P. aeruginosa with homology ranging from (90-95.77%) when compared to already registered and listed in the GenBank bacterial strains. Most isolates showed high resistance degrees to ampicillin, ampicillin/sulbactam, clindamycin, tetracycline, and rifampin, nalidixic acid, streptomycin, ceftriaxone, gentamycin, cefotaxime, ceftazidime, pipercilin, tobramycin, and ciprofloxacin. It was moderately resistant to norfloxacin, meropenem, imipenem, pipracilin, and tazobactam.

Keywords: Pseudomonas aeruginosa, antimicrobial susceptibility, 16S rRNA gene, PCR.

1. INTRODUCTION

P. aeruginosa is an aerobic gram-negative bacillus that is the second most prevalent cause of infection in hospital wards, including critical care units. Due to the extended spread of P. aeruginosa habitat, the control of the organism in a hospital setting is very difficult and makes it practically impossible to prevent contamination [1]. This organism develops resistance to antimicrobial medicines in a variety of ways, including changing the microorganism's permeability to medications; enzymatic resistance; efflux pump; drug receptor alteration; and developing a secondary metabolic pathway [2]. As a result of the repeated indiscriminate use of antibiotics to treat cases of Pseudomonas infection, the problem of antibiotic resistance has emerged in this bacteria. Some of the causes of resistance to antibiotics arise in this bacteria by mutation or additional horizontal transmission via conjugation by plasmids and transposons containing antibiotic resistance genes [2, 3].

Therefore, health workers have developed modern methods for discovering pathogens in addition to the traditional methods previously used, which are phenotypic identification of microbes using microscopy, culture, and biochemical tests, which are still used even today for the diagnosis of diseases. However, these methods of bacterial identification have major weaknesses [4].

For starters, they cannot be used to cultivate non-cultivable organisms. Second, we occasionally encounter organisms with biochemical traits that do not match those of any known species or genera. Third, identification of slow-growing organisms would be extremely slow and difficult [4]. Therefore, the CLSI (Clinical and Laboratory Standards Institute) guideline suggests 16S rRNA for the diagnosis of diseases. 16S rRNA gene sequences to study bacterial phylogeny and taxonomy have been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (approximately 1,5 kb) is large enough for informatics purposes [5].

Despite its accuracy, Due to financial and technical constraints, 16S rRNA gene sequence analysis is not commonly used outside of big and reference facilities. In this article, the bacterial 16S ribosomal RNA gene was used for PCR amplification and subsequent sequencing analysis of the 16S ribosomal RNA (rRNA) gene has been widely used to identify bacterial species. The 16S rRNA gene of bacteria typically contains nine "hypervariable areas" that show significant sequence variety among various bacterial species and can be utilized to identify species. These hypervariable regions are flanked by conserved stretches in most bacteria, enabling PCR amplification of target sequences using universal primers [6].

The goal of this study was to identify P. aeruginosa isolated from clinical specimens (burns and wounds) in Al Muthanna hospitals using traditional methods and to sequence the 16S rRNA gene by PCR amplification technique in order to evaluate the utility of newly designed 16S rRNA primers in a clinical microbiology laboratory by comparing them to identification using universal PCR primers and phenotypic methods. Also, the study aims to investigate the antimicrobial resistance pattern of P. aeruginosa isolated to a variety of antibiotics by the Kirby-Power Disc Diffusion method.

2. Materials and Methods

2.1 Bacterial isolation and identification

The study included the collection of 100 swabs from clinical samples (burns and wounds) in Al Muthanna hospitals during the period from November 2021 to March 2022. All samples were collected by using sterile cotton swabs after cultured on the brain heart infusion broth media for the purpose of activating the bacteria because this medium is considered an enriched medium, and it was incubated for 24 hours at a temperature of 37°C. Then the samples were cultured on Blood agar and MacConkey agar and incubated at 37°C for 24 hours, and extended to 48 hours if no growth appeared. Then they were further sub cultured overnight at 37°C on selective medium, chrome agar, and HiFluoro pseudomonas agar base, showing pure colonies on this selective medium. After that, microscopic examination and biochemical tests are performed on these colonies to ensure the presence of P. aeruginosa. Purified colonies were first identified through bacteriological reactions and cultural traits [7].

2.2 Antibiotic susceptibility testing of bacterial isolates

The antibiotic susceptibility test was carried out using the Kirby-Bauer disc diffusion technique, as described in [8,9]. The Clinical and Laboratory Standards Institute [10] provides a reference. Amikacin AK (30mcg), tobramycin TOP (10mcg), and streptomycin (S-25mcg), aztreonam AT (30mcg), imipenem IMP (10mcg), and meropenem MRP (10mcg), clindamycin DA (10MCG), tetracycline TE (30mcg), rifampin. In brief, an overnight suspension broth culture matched to 0.5 MacFarland Standard was uniformly inoculated by cotton swab across the whole surface of Muller-Hinton agar and incubated at 37°C for 24 hours. Dishes were afterwards read by measuring the widths of the growth inhibition zones (millimetres) surrounding each antibiotic disc. The drug zone diameter was calculated using Clinical and Laboratory Standards Institute guidelines [10].

2.3 DNA extraction

All suspected isolates were cultured in BHI broth for 24 hours at 37 degrees Celsius. Three microliters of overnight culture were centrifuged at 14000 g for two minutes. After discarding the supernatant, the pelleted cells were collected. Following the manufacturer's instructions, bacterial DNA was obtained using the FAVORGEN company's Genomic DNA extraction kit and used as a DNA template for the following PCR experiment.

2.4 Detection 16S rRNA gene by conventional PCR of P. aeruginosa

For complete detection of isolates, randomly selected 12 isolates only for DNA sequencing as templates for PCR amplification of the 16S rRNA gene due to the limitation of resources. The two primers used were chosen according to [11] were 27F-5-AGAGTTTGATCCTGGCTCAG-3 and 11392R-5-GGTTACCTTGTTACGACTT-3 for forward and reverse primers (Macrogen, South Korea) was used to amplify a specific region of previously extracted DNA samples to confirm and identify the isolates of P. aeruginosa. The PCR reaction was completed in 50µl reaction mixtures by using the 25µl GoTaq® G2 Green Master Mix, 2X solution (Promega Company, USA) with 5µl for each primer of 10 pmol of each primer, and 5µl of DNA template. The final volume was adjusted by adding 10µl of nucleus-free water to each reaction tube. The PCR conditions were initial denaturation of 95°C for 5 min, followed by 30 cycles of denaturation of 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. Gel electrophoresis in 1.0% agarose stained with ethidium bromide was used to examine PCR products. The results were photographed under an ultraviolet light machine to detect the specific amplified product by comparing it with a 100-5000bp standard DNA ladder (figure 2). The remains from PCR products were stored at -20°C until sequencing.

2.5 16S rRNA Gene Sequencing and Phylogenetic Analysis

The eight PCR products were then sequenced by Macrogen Corporation-Korea using ABI3730XL, an automated DNA sequencing system. The blast analysis is then used to distinguish a comparable sequence in the https://www.ncbi.nlm.nih.gov NCBI database. The phylogenetic tree was constructed by the neighbor-joining method using the Geneious 7.2.2 (Biomatters) software.

3. Results

3.1 Prevalence of isolations and identification of P. aeruginosa

A total of 55 clinical isolates were identified as P. aeruginosa by traditional methods, including growth characteristics, colony morphology, and biochemical tests. P. aeruginosa was identified as Gram negative rods, lactose nonterminating, appearing as large colonies with a characteristic grape-like odour as shown in Figures 1. The 16SrRNA in the isolates was amplified by PCR. In total, 8 were sent for characterization by sequencing of PCR products, and when the gel electrophoresis is performed for DNA screening of isolates, the results show the appearance of bands of 1500 bp compared with a 5000 bp DNA ladder. On the basis of the similarity and difference between the samples and based on the results of gel electrophoresis of PCR results for gene 16S rRNA shown in Figure 2. Sequence analysis by the BLAST tool was used to compare them to the existing global sequences in GenBank, which showed a high similarity of isolation with 96%, which confirmed the biochemical diagnosis of the isolate, and Table 1 shows the rate of convergence in the diagnosis with the nearest 8 reference isolates, based on identity. Turkey (HQ377326.1), Pakistan (MF977350.1, MF802727.1), and Malaysia (MG650162.1, MN938189.1, KT270317.1, EU734822.1, JX393834.1, KF835840.1). The connection between examination sequences and their adjacent relatives was evaluated using the Geneious software to form the phylogenetic tree, as shown in Figure 3. Concerning antibiotic susceptibility test, generally, all isolates exhibited relatively varied resistance rates against 20 different antibiotic. In general, the P. aeruginosa isolates (100%) were resistant to ampicillin, ampicillin/sulbactam, clindamycin, tetracycline, and rifampicin, and at a percentage of 94.54% against nalidixic acid. On the other hand, most of the isolates showed high resistance against some antibiotics, such as streptomycin 89.09%, ceftriaxone 87.27%, cefotaxime and gentamicin were 83.63% and resistant to ceftazidime 80%, followed by 78.18% for pipercilin and tobramycin, and resistant to monobactam was moderate, which showed a 54.54% resistant level to aztreonam, followed by 49.09% and 36.36% for ciprofloxacin and norfloxacin respectively, while the lowest resistance effect was observed for carbapenems such as meropenem at 27.27% and imipenem at 18.18%. Furthermore, some antibiotics had the lowest effect for β-lactamase inhibitor combination agents, such as imipenem/EDTA at 5.45% and pipracilin/tazobactam at 9.09%.

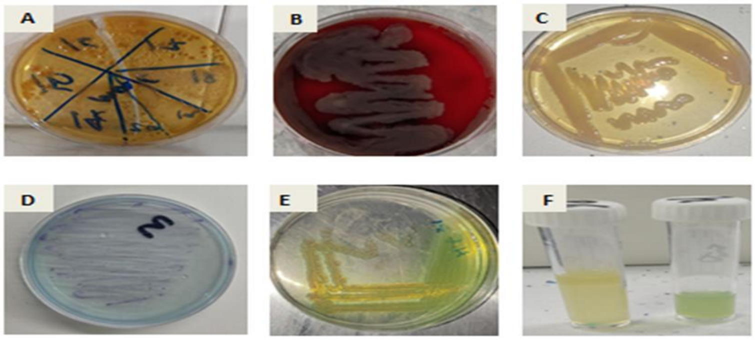

Figure 1: Pseudomonas aeruginosa Cultured (37°C for 24 hrs) on: (A) MacConky agar (B) Blood agar (C) Nutrient agar (D) Pseudomonas Chrome agar, (E) HiFluoro™ Pseudomonas Agar Base (F) Brain heart infusion broth.

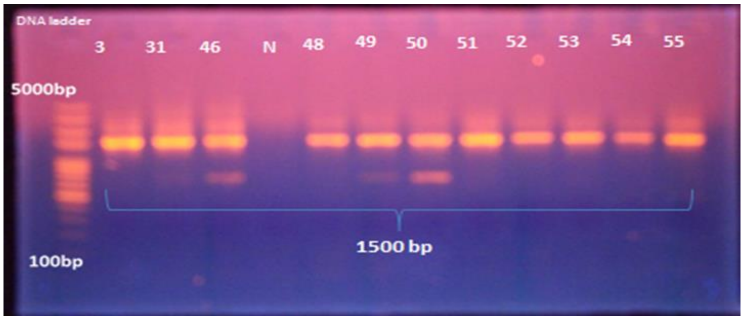

Figure 2: Agarose Gel electrophoresis of amplified PCR product for the detection of 16srRNA run on 1%agarose (90 min at 70 volt), stained with ethidium bromide lane 3,31,46,48,49,50,51,52,53,54,55 sample P. aeruginosa isolates: Marker DNA ladder (100-5000)bp. note (N) negative control.

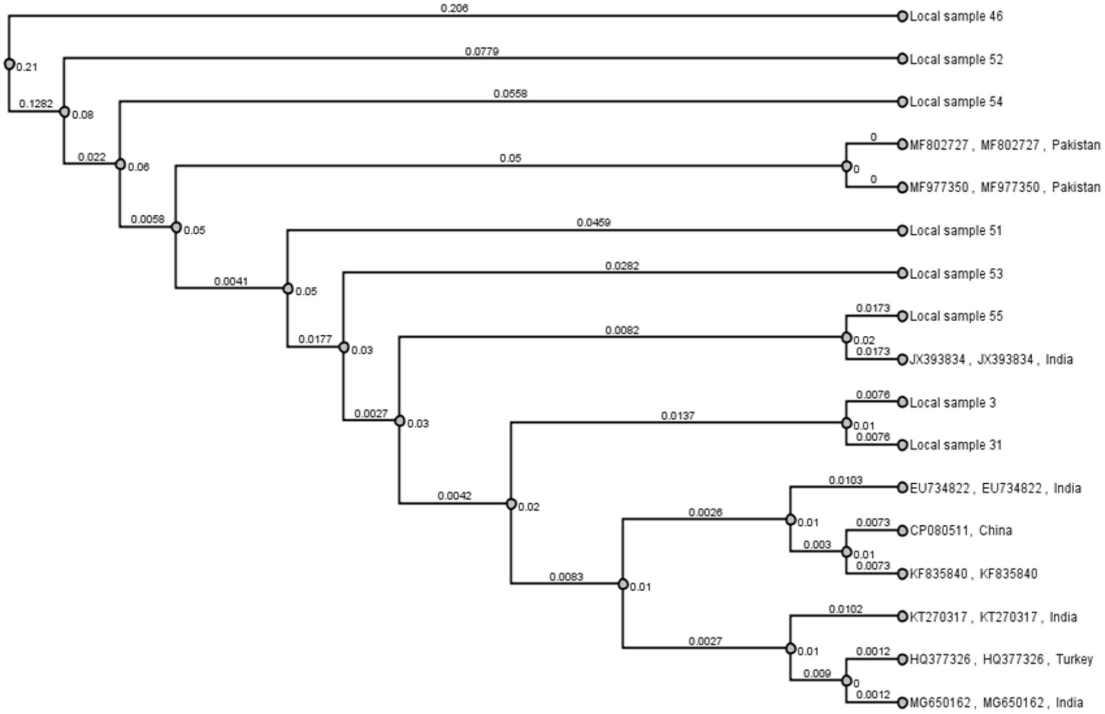

Figure 3: Phylogenetic relationships of Pseudomonas aeruginosa isolated from wound and burn infections in Iraq and with other international strains which were extracted from GenBank database.

4. Discussion

In particular, about 55 (68.75%) isolates of P. aeruginosa were diagnosed from burns and wound infections in Al Muthanna hospitals. A similar study done in Duhok city, Iraq found sixty (11.4 %) P. aeriginosa isolates were diagnosed from burns and wound infections [12]. Other studies in Iraq found 31 P. aeruginosa isolates from educational Al-Yarmouk and AL Kadhimiya hospitals in Baghdad [13]. These differences and diversity in proportions might be due to the type of inclusion criteria for enrolled patients such as gender, specimen types, kind of test used in isolation and usage of preventive handling that determines the percentage [14]. The present results agree with [15] who reported that 16S rRNA gene sequencing is now common in medical microbiology as a quick and inexpensive alternative to phenotypic approaches for bacterial identification. To complete the genetic detection of P. aeruginosa, the results of sequences had to be analysed using the (BLAST) to form a comparison with NCBI information data and to determine the differences in the sequences, as shown in Table 1, which shows the samples numbers and how they correspond to a reference strain and their accession numbers ID. Only 10 local isolates had 96 % similarity to the detected P. aeruginosa strain recoded earlier in the GenBank, according to the sequencing results in Table 1. The rest of the local analysis samples were also recognised as P. aeruginosa with homology ranging from (90-95.77%) when compared to already registered and listed in the GenBank bacterial strains, Furthermore, differences in the identified results between query and subject sequences could be attributed to horizontal gene transfer in all molecular studies, as P. aeruginosa is pathogenic bacteria that can change its genetic materials to better survive and adapt to a new environment. In addition, the Geneious software was used to investigate the phylogenetic tree, which was constructed using the nearby technique. Furthermore, the Geneious software was used to automatically eliminate all locations with gaps and missing data. The bacterial strains were divided into three primary separated groups with high bootstrap percentages, corresponding to unique strains, as shown in Figure 3. The first group shows the distribution of local samples 46,52,54, most likely sharing an ancestor and belonging to the same branch as the Pakistan samples. The second set shows the local samples 51,53,55 likely sharing an ancestor and belonging to the same branch as the Pakistan and India samples. The three sets show the local samples 3, 31 likely sharing an ancestor and belonging to the same branch as the India, China, and Turkey samples. According to the results of the phylogenetic tree, they shared a similar sequence of the 16S rRNA gene, which is used in the classification of bacteria around the world because it is highly conserved, has a low mutation rate, and takes a long time to change. Despite the fact that the absolute rate of change in the 16S rRNA gene sequence is unknown, these characteristics do not preclude or limit the use of the 16S rRNA gene sequence for bacterial identification and taxonomic studies [16].

Table 1: The identification results of 8 strains of Pseudomonas aeruginosa based on 16S rRNA Sequences analysis

NO	**Sources**	**Identities**	**GenBank ID**	**Country**
3	*Pseudomonas aeruginosa strain ABL17CA29*	96%	MG650162.1	India
	Pseudomonas aeruginosa strain DJ06	96%	CP080511.1	China
	Pseudomonas aeruginosa strain On7	96%	HQ377326.1	Turkey
31	*Pseudomonas aeruginosa strain ABL17CA29*	96%	MG650162.1	India
	Pseudomonas aeruginosa strain DJ06	96%	CP080511.1	China
	Pseudomonas aeruginosa strain IR1	96%	KT270317.1	India
	Pseudomonas aeruginosa strain NBRAJG90	94.5%	EU734822.1	India
	Pseudomonas aeruginosa strain SKN2	94.35%	JX393834.1	India
	Pseudomonas aeruginosa strain Zp3	94.5%	KF835840.1	India
46	*Pseudomonas aeruginosa strain ABL17CA29*	96%	MG650162.1	India
	Pseudomonas aeruginosa strain LS-1	90%	MF977350.1	Pakistan
	Pseudomonas aeruginosa strain OLVS-1	90%	MF802727.1	Pakistan
51	*Pseudomonas aeruginosa strain ABL17CA29*	90%	MG650162.1	India
	Pseudomonas aeruginosa strain LS-1	90%	MF977350.1	Pakistan
	Pseudomonas aeruginosa strain OLVS-1	90%	MF802727.1	Pakistan
52	*Pseudomonas aeruginosa strain ABL17CA29*	96%	MG650162.1	India
	Pseudomonas aeruginosa strain DJ06	96%	CP080511.1	China
53	*Pseudomonas aeruginosa strain ABL17CA29*	95.57%	MG650162.1	India
	Pseudomonas aeruginosa strain DJ06	95.12%	CP080511.1	China
	Pseudomonas aeruginosa strain NBRAJG90	94.46%	EU734822.1	India
	Pseudomonas aeruginosa strain SKN2	94.42%	JX393834.1	India
	Pseudomonas aeruginosa strain IR1	93.99%	KT270317.1	India
	Pseudomonas aeruginosa strain Zp3	94.84%	KF835840.1	India
	Pseudomonas aeruginosa strain On7	94.86	HQ377326.1	Turkey

54	*Pseudomonas aeruginosa strain ABL17CA29* *Pseudomonas aeruginosa strain DJ06* *Pseudomonas aeruginosa strain IR1*	96% 96% 94%	MG650162.1 CP080511.1 MN938189.1	India China India
55	*Pseudomonas aeruginosa strain NBRAJG90* *Pseudomonas aeruginosa strain SKN2* *Pseudomonas aeruginosa strain IR1* *Pseudomonas aeruginosa strain ABL17CA29*	96.29% 95.77% 95.77% 95.33%	EU734822.1 JX393834.1 KT270317.1 MG650162.1	India China India India

Antimicrobial resistance (AMR) has become a significant problem due to the growing impact on healthcare systems worldwide. Due to prolonged hospitalization, antibiotic resistance has recently been related to high rates of morbidity and mortality. Antibiotic resistance is a normal occurrence in bacteria, but it is implied when antibiotics are misused in humans and animals [17]. The emergence of the problem of antibiotic resistance in P. aeruginosa isolates poses a public health threat. In the current study, the resistance rates against different antibiotics were variable. The 55 isolates showed the highest resistance against ampicillin, ampicillin/sulbactam, tetracycline and clindamycin in percentage (100%) these results agreement with a previous study by [18,19] but not consistent with [20] if he obtained a tetracycline resistance rate of 17.5%. Additionally, there was substantial aminoglycoside resistance such as streptomycin at 89.09%, gentamycin at 83.63%, and tobramycin at 78.18%, these results were in agreement with [18,21] in Kirkuk and in Basrah City. While the rates of resistance to ceftriaxone were 87.27%, cefotaxime 83.63%, and ceftazidime 80%, this result agreed with [22]. Furthermore, resistant to monobactam was moderate, which showed a 54.54% resistant level to aztreonam, followed by 49.09% and 36.36% for ciprofloxacin and norfloxacin respectively, these results agree with a study [23,24,25] in Wasit and Baghdad hospitals in Iraq.. Additionally, about 78.18% of the isolates were found to be resistant to piperacillin. This result is consistent with the value reported by [24]. The results of our study showed a percentage of 9.09% of P. aeruginosa isolated is resistant to pipracilin/tazobactam this finding was consistent with [26] Carbapenems are considered the most significant group of antibiotics against MDR P. aeruginosa, but the development of carbapenem resistance is becoming a challenge for health care professionals and has limited therapeutic options. Sufficient measures are required to prevent the spread of the carbapenemase encoding gene [27]. The current study demonstrated that the resistance for both meropenem and imipenem was 27.27% and 18.18% respectively, this finding is consistent with [28,29]. However, results disagree with results [30], who recorded that toxA gene polymorphism showed the acquisition of high meropenem resistance. The results of the sensitivity test showed about 5.45% were resistant to the combined imipenem-EDTA disc method, which was performed for detection of the ability of P. aeruginosa isolates to produce MBL because EDTA causes destruction of the outer membrane of the bacterial cell, altering the permeability to antibiotics that enter the bacterial cell and exert their effect [31]. The results of the current study showed that there is common resistance among other classes of antibiotics such as pencillins, cephalosporins, monobactam, fluoroquinolones, aminoglycosides, tetracyclines, and clindamycin. It is considered a multidrug-resistant (MDR) strain.

5. Conclusions:

Identification of this species may be problematic due to the marked phenotypic variability demonstrated by sample isolates and the presence of other closely related species. To facilitate species identification, we used 16S ribosomal (rRNA) sequence data to identify genus- and species-specific 16S rRNA signature sequences, which are accounts a stable part of the genetic code. Based on these sequences, we designed simple, rapid, and accurate PCR assays that allow the differentiation of P. aeruginosa from Pseudomonas species and other pathogen genuses.

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