RESEARCH ARTICLE

Identification of Clinical *Pseudomonas* spp. by VITEK 2 Compact System and Species-specific Polymerase Chain Reaction Assay for Identification of *Pseudomonas aeruginosa*

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ABSTRACT

The objective of this study was to isolate, identify, and diagnose *Pseudomonas* spp. from different clinical sources in Baghdad, Iraq. VITEK 2 compact system identification gram-negative bacteria (ID gNB) cards were used to confirm the identification. Polymerase chain reaction (PCR) technique and sequencing were used for recognition of the 16S rDNA gene, by two pairs of primers, universal primers (930 bp fragments) for recognition of *Pseudomonas* spp., and *Pseudomonas aeruginosa* specific species (PASS) primers (956 bp fragments) for differentiation of *P. aeruginosa* from other species. Amplified PCR products of PASS primers were sent for DNA Sanger sequencing to Macrogen Company, Seoul, Korea; data were compared with the database using the Basic Local Alignment Search Tool (BLAST). Ninety-two *Pseudomonas* spp., including 86 isolates of *P. aeruginosa* and 1, 2, 3 isolates of *Pseudomonas luteola, Pseudomonas putida*, and *Pseudomonas fluorescens*, respectively, were obtained using VITEK 2 compact system ID gNB cards with a percentage of identification ranging from 91 to 99%. Gene amplification and sequencing results confirm identification ranging from 99 to 100%. After sequencing analysis, eleventh of the *P. aeruginosa* isolates had been submitted in GenBank National Centre for Biotechnology Information (NCBI) under accession numbers MN630696–MN630707. It was observed that phenotypic tests supported by PCR techniques have enabled to conduct a detailed characterization of *Pseudomonas* bacteria isolates.

Keywords: *Pseudomonas aeruginosa*, *Pseudomonas* spp., Species-specific polymerase chain reaction (PCR) assay.

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INTRODUCTION

The genus *Pseudomonas*, of the Pseudomonadaceae family, has a leading role among infection caused by gram-negative rods. *Pseudomonas* bacteria are motile gram-negative aerobic bacteria, 2 to 4 µm long plump shaped rods, with polar flagella. Nearly all common species of *Pseudomonas*, which have associated with humans can cause disease, such as, eye infection, bacteremia, and endocarditis. Among the genus *Pseudomonas*, there are many species, including *P. aeruginosa*, *P. putida*, *P. luteola*, and *P. fluorescens.*[1-3] *P. putida* is a rare reason for infection in humans. However, there are numerous reports of wound and eye infections, bacteremia, urinary tract infection, central venous catheter infection, pneumonia, and soft tissue infections caused by *P. putida*.[4-8] *P. luteola* may cause infections of the bloodstream associated with pancreatitis, intravenous indwelling catheters, prosthetic valve endocarditic, foreign bodies, and coetaneous abscesses. Infrequently, non-bacteremia cases have been reported as post neurosurgical infections, fatal meningitis, appendicitis, or peritoneal dialysis catheters femur abscess, endophthalmitis, facial cellulite, leg ulcer in a patient with the sickle disease, peritonitis complicating, subphrenic abscess, and hand infection.[9-11] There are a number of reports recognizing *P. fluorescens* as a human pathogen. There is an exciting association between *P. fluorescens* and human disease, in that approximately 50% of Cohn's disease patients develop serum antibodies to *P. fluorescens*.[12] It was found out that *P. aeruginosa* remains the most frequent etiology of the intensive care unit,[13] and is one of the major pathogens causing hospital-acquired infections because it can easily develop antibiotic resistance.[14] To identify any bacterial isolate, several methods had to be applied; phenotypic tests are expensive, and time- and labor-consuming. Sometimes, the results of the identification of some strains are ambiguous to interpret, so the genetic technique supports the phenotypic test. Presently, genetic techniques supported phenotypic tests enable

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Table 1: PCR primers that are used for genotypic detection of bacterial isolates

Primers of 16S rDNA	*Primer sequence (5`-3`)*	*Product size (bp)*	*Reference*
27-F	5`- AGAGTTTGATCCTGGCTCAG-3`	930	18
1492-R	5`-CTTGTGCGGGCCCCCCGTCAATTC-3`		
PASS-F	5`-GGGGGATCTTCGGACCTCA-3`	956	19
PASS-R	5`-TCCTTAGAGTGCCCACCCG-3`		

to conduct the characteristic of *Pseudomonas* strains isolated from different sources. A study by Fernández-Olmos A *et al.*[15] identified *Bukholderia cepacia* and *Pandoraea* spp. by using the PCR technique for detection of the 16S rDNA gene, in a study by Maheswarappa G *et al.*[16], they used the sequencing of *P. aeruginosa* strains product to find the similarities between them. This study aims to differentiate between clinical *Pseudomonas* spp. Strain, using phenotypic and molecular methods, and to deposit some of them in the GenBank (NCBI).

MATERIALS AND METHODS

Specimen Collection

Two hundred fifty different clinical specimens were collected from different Baghdad hospitals during the period from November 2018 to February 2019.

Identification of Clinical Specimens

All of the specimens were cultured on MacConkey agar medium and incubated aerobically at 37ºC for 24 hours. For the primary isolation, the suspected non-lactose fermented colonies grown on MacConkey agar medium were cultured on the cetrimide agar medium at 37ºC for 24 to 48 hours. Cetrimide agar is the recommended medium for the primary identification and isolation of *P. aeruginosa*. All suspected isolates obtained on cetrimide agar medium were cultured on brain heart infusion agar, and the isolates were furtherly characterized by performing gram staining, and some conventional biochemical tests, like oxidase and catalase production, growth at 42ºC,[17] and to confirm the isolation VITEK 2 system compact ID gNB cards were used.

PCR and 16S rDNA Gene Sequencing Identification Method

Extraction of Genomic DNA

Bacterial genomic DNA was extracted by using a commercial purification system [Wizard® genomic DNA purification kit (Promega, USA)].

Preparation of Primers

The primers (Alpha DNA, Canada) were provided in lyophilized form. These primers were dissolved in deionized distilled water to give a final concentration, 100 picomol/µL, as recommended by the provider company as a stock solution, which kept in a deep freezer (-20ºC) until use. Table 1 shows the primers used in our study.

PCR Amplification of 16S rDNA Gene

The component of the PCR reaction in this study is shown in Table 2.

Table 2: Component of PCR technique

Component	*Volume (µL)*
PCR Master Mix 2x	12.5
Forward primer (10 pmol/µL)	1
Reverse primer (10 pmol/µL)	1
DNA template	2
Nuclease free water	8.5
Final volume	25

Table 3: Amplification program of PCR by universal primers

Function	*Temperature (ºC)*	*Time*	*Cycle*
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	
Annealing	55	30 sec	30
Extension	72	1 min	
Final extension	72	10 min	1

Table 4: Amplification program used in thermocycler PCR by PASS primers

Function	*Temperature (ºC)*	*Time*	*Cycle*
Initial denaturation	95	2 min	1
Denaturation	94	20 sec	
Annealing	58	20 sec	25
Extension	72	40 sec	
Final extension	72	1 min	1

Amplification was done as described in Table 3[18] for universal primers (27-F and 1492-R).

The program that was used in thermocycler PCR for PASS primers was performed according to Spilker T *et al.*[19]. The steps of the program are summarized in Table 4.

Agarose Gel Electrophoresis

After PCR amplification was completed, agarose gel electrophoresis was adopted to prove the presence of amplification. PCR was totally dependable on the extracted DNA criteria.

Preparation of Agarose

One-gram of agarose powder was added to 100 mL of (1X) tris-borate-ethylenediaminetetraacetic acid (TBE) buffer in a glass beaker, and then the solution was heated till arriving at boiling by microwaving for about 1.3 seconds, until the agarose was dissolved totally. Then, the solution was left to cool at 45ºC, then 5 µL of RedSafe solution (10 mg/mL) was added to enable the visualization. Then, the prepared agarose solution was poured into the gel griddle, which contains two combs. After sealing the two edges of the agarose, it was left to solidify at

room temperature. Then, the comb was removed carefully, leaving wells on the gel. The wells were filled with 7 µL of the extracted DNA sample and were mixed with 3 µL of loading dye, while for PCR, each well was loaded with 10 µL from samples. A 100 bp DNA ladder was served as a size marker, always run currently with each electrophoresis, which runs to detect the product size of the PCR product.

Gel Electrophoresis

After the PCR, product 10 µL was loaded into the gel well, and 5 µL of DNA ladder (100 bp) was loaded to serve as a marker during electrophoresis work. The gel was located into the gel chamber of the gel electrophoresis apparatus, which was full with 1X of T.B.E. buffer. Then, the chamber was closed, and electrophoresis runs for 30 to 40 minutes at 135 volts. Then, the agarose gel was detached from the chamber, and the gene bands were visualized by UV transilluminator and photographed.[20]

Sequencing Analysis of PASS Primers

Amplified PCR products of PASS primers were carried out by the Macrogen DNA sequencing company (Seoul, Korea) by the dideoxynucleotide chain-termination method (Sanger method). DNA sequences were analyzed and compared with already known sequences using the BLAST NCBI website.

RESULTS AND DISCUSSION

Isolation and Identification of *Pseudomonas* spp.

92 isolates of *Pseudomonas* spp. including 8 (93.47%) *P. aeruginosa*, 3 (3.26%) *P. fluorescence*, 2 (2.17%) *P. putida*, 1 (1.08%) *P. luteola*, were obtained during this study.

Distribution of *Pseudomonas* spp. according to Clinical Source

The sources of *Pseudomonas* spp. are shown in Figure 1.

Distribution of *Pseudomonas* spp. according to Age

The distribution of *Pseudomonas* spp. according to age is shown in Figure 2.

Distribution of *Pseudomonas* spp. according to Sex

The distribution of *Pseudomonas* spp. according to sex is shown in Figure 3.

Gram stain showed single cells, rod shapes, and gram-negative rods; these results agreed with Quinn PJ *et al.*[21] and Høiby N *et al.*[22]

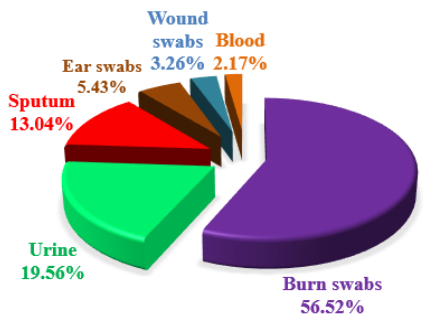

Figure 1: Distribution of *Pseudomonas* spp. isolates according to clinical sources

On MacConkey agar medium, *P. aeruginosa* colonies appeared small, round, convex, rough colony with irregular edges, whitish, or creamy in color (lactose non-fermenting), and has a fruity odor.[23]

Clear zone appeared around the beta hemolysin colonies on blood agar medium. These results are in agreement with Quinn PJ *et al.*[21] and Mbajiuka S *et al.*[24]

The *P. aeruginosa* isolates were identified successfully on cetrimide agar medium. This medium is a selective medium for the identification of *P. aeruginosa*.[25]

For the characterization of *Pseudomonas* spp., different biochemical tests were performed (Figure 4-9). The bacterial isolates were positive for oxidase, catalase, and growth at 42ºC, while the negative result for the indole test, methyl red test, methyl red -voges proskauer (MR-VP) test, gram stain, citrate utilization, and urease test.[26-28]

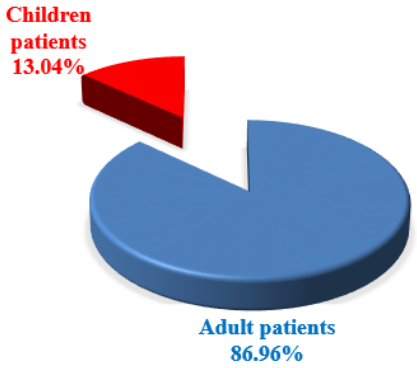

Figure 2: Distribution of *Pseudomonas* spp. according to age

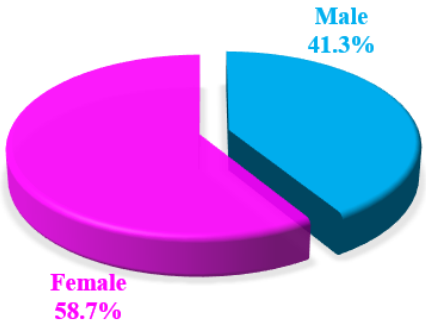

Figure 3: Distribution of *Pseudomonas* spp. according to sex

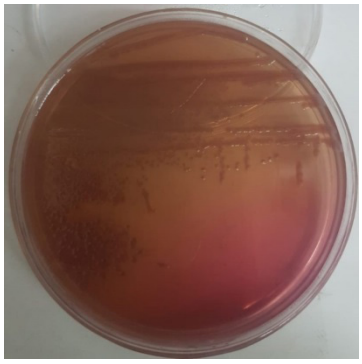

Figure 4: *Pseudomonas* spp. isolates on MacConkey agar medium

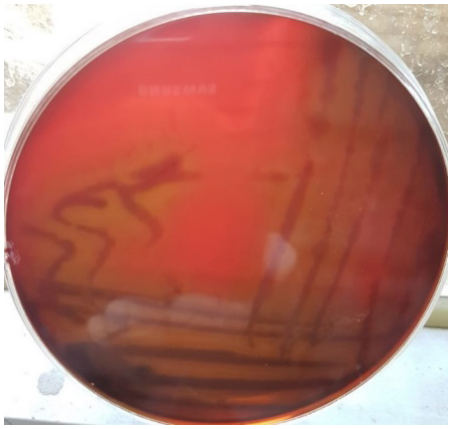

Figure 5: Hemolysin production by *P. aeruginosa*

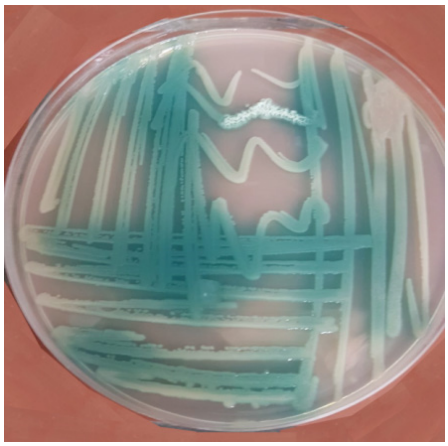

Figure 6: *P.aeruginosa* on cetrimide agar medium

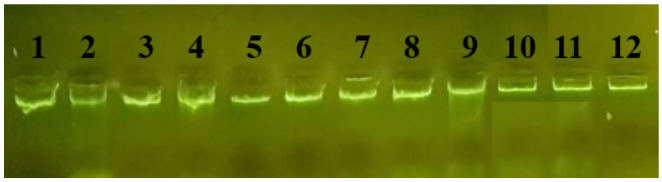

Figure 7: Analysis of genomic DNA from isolates on agarose gel (1%) at 135 V for 30 minutes under UV light, after staining with red safe dye; lane 1: *P. luteola*; lane 4, 5, 6: *P. flourescense*; lane 11: *P. putida*; lane 2, 3, 7, 8, 9, 10, 12: *P. aeruginosa*

Table 5: Identification of *P. aeruginosa* isolated based on PASS primers of 16S rDNA sequence and NCBI database

Isolate No.	*Percentage of similarity (%)*	*Identified as*
12	99	*P. aeruginosa*
13	100	*P. aeruginosa*
14	100	*P. aeruginosa*
28	100	*P. aeruginosa*
56	100	*P. aeruginosa*
67	99	*P. aeruginosa*
75	99	*P. aeruginosa*
78	100	*P. aeruginosa*
79	100	*P. aeruginosa*
83	100	*P. aeruginosa*
85	100	*P. aeruginosa*

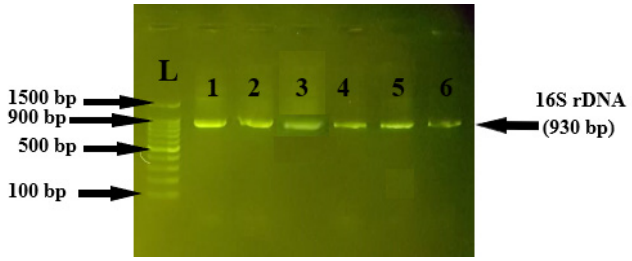

Figure 8: Agarose gel electrophoresis of universal primers of 16S rDNA gene PCR product (930 bp); lane L: 100 bp DNA ladder; lanes 1–6: represent *Pseudomonas* spp. isolates no. 1, 4, 5, 6, 11, 20, respectively; electrophoresis was done on agarose gel (1%) at 135 V for 30 minutes, under UV light after staining with red safe dye

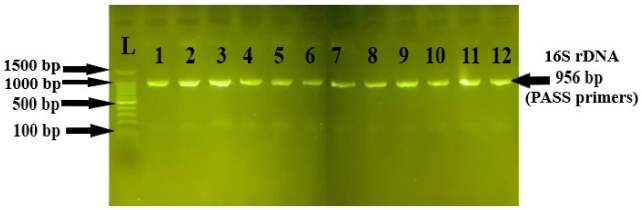

Figure 9: Agarose gel electrophoresis for detection of PASS primers of 16S rDNA gene PCR product (956 bp); lane L: 100 bp DNA ladder; lanes 1–12: represent *P. aeruginosa*; electrophoresis was done on agarose gel (1%) at 135 V for 30 minutes, under UV light after staining with red safe dye

Molecular Identification of *Pseudomonas* spp. Isolates by 16S rDNA Gene and Sequence Analysis

Genomic DNA was successfully extracted from all 92 of *Pseudomonas* spp. isolates under study, by using a commercial genomic DNA purification kit, to detect 16S rDNA gene.

Each DNA extracted samples was subjected to PCR reaction. The PCR products were analyzed by agarose gel electrophoresis. The length of amplified fragments was 930 bp of universal primers, and 956 bp of PASS primers for the 16S rDNA gene.

The PCR product from the *P.aeruginos*a isolates was sequenced by DNA sequencer, and the sequence data compared using the BLAST program in NCBI, by which the PASS primers of 16S rDNA was aligned. The sequence analysis of these isolates showed strong similarities with the representative isolates that were retrieved from the NCBI database, and also determined the phylogenetic affiliation, based on nucleotide homology and phylogenetic analysis. The identification and similarity of selected isolate with other *P. aeruginosa* isolates in the gene bank database (NBCI) is shown in Table 5.

Previous study by Spilker T *et al.*[19] for differentiation of *P. aeruginosa* from another *Pseudomonas* spp., recovered from cystic fibrosis patients based on the arrangement of 16S rDNA sequences available in GenBank, the two primer pairs were designed. Primer pair PA-GS-F and PA-GS-R was planned to amplify all *Pseudomonas* species, while the pair PA-SS-F and PA-SS-R was planned to amplify only *P. aeruginosa*. Various studies were performed using 16S rDNA for the identification of various bacteria. A study conducted by Bosshard PP *et al.*[29]

used 16S rRNA gene sequence for the identification of gram-negative bacilli. A study conducted in Iraq used the PCR technique for the detection of 16S rDNA gene for identifying *P. aeruginosa*.[30] Study conducted by Azeez B *et al.*[31] identified *P. aeruginosa* by using the PCR technique for the detection of 16S rRNA. A local study by Hasson NA *et al.*[32] used PASS primers of 16S rDNA for differentiation of *P. aeruginosa* from other bacteria.

CONCLUSION

Our study proves that various non-aeruginosa pseudomonal species can be recovered from clinical sources. 16S rDNA gene sequencing demonstrates its utility in the identification of *Pseudomonas* spp. from clinical sources.

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