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Molecular identification of *Pseudomonas aeruginosa* isolated from Hospitals in Kurdistan region

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ABSTRACT

Pseudomonas aeruginosa is an aerobic Gram-negative bacterium which has emerged as one of the most problematic nosocomial pathogens. To characterize *P. aeruginosa* strains that are widespread in patients in Kurdistan region of Iraq, 165 clinical samples were collected from wounds, burn, ear infection and urinary tract infection taken from general hospitals of different areas of the region. Methods for isolation and identifying *P. aeruginosa* based upon culture methods coupled with biochemical tests, were used in this study. The results show that, the selective medium (cetrimide agar) at 42°C aerobically had highest recovery in the isolation of *P. aeruginosa* strains, they were produced greenish-yellow or blue pigment colonies, catalase and oxidase was positive whereas negative for methyl red, Voges Proskauer and indole.; however, some of these methods are time consuming and may not be very accurate whereas API 20E is rapid method which performs at least 20 different biochemical tests at once, however it proved difficult to obtain additional information concerning the relationship between these strains. To study the susceptibility of tested strain to different antibiotics using disk diffusion the result show that all the strains of *P. aeruginosa* were resistance to Cefotaxime, Chloramphenicol, Clindomycin, Erythromycin, Gentamycin, Nitrofurantoin and Pincillin, and susceptible to Ciprofloxacin, Imipenin, Lincomycin, Piperacillin and Tobramycin, while the strains showed differs susceptibility for Amikacin, Nalidixic acid, Rifampicin, Tetracycline and Trimethoprim.. In attempting to identification of *P. aeruginosa* strains at the DNA level, Polymerase chain reaction (PCR) is used based on specific primer for 16S rRNA and OPRL gene, the results showed that PCR has found to be rapid and more sensitive and specific in identification of *P. aeruginosa*, however, combination of more than one specific marker is recommended.

Keywords: Biochemical tests, *Pseudomonas aeruginosa*, PCR, susceptibility to antibiotic.

1. Introduction

P. aeruginosa has emerged as one of the most problematic nosocomial pathogens, it is considered an opportunistic pathogen that causes infection in immune depressed subjects (Brooks *et al.*, 2007). It is the leading cause of wound infections, urinary tract, surgical wound and ear infection (Todar, 2008). A rapid and accurate system for the identification of *Pseudomonas* is important to isolate patients and prevent further spreading of the diseases. Cultivation of bacteria is one of the most important techniques which still used in diagnostic microbiology, because of its ability to quantify the viable bacteria in a sample; as well as obtaining a pure sample for further testing (Minion, 2010). Many studies satisfied by using the API 20E system or classical biochemical test for bacterial identification (Capuzzo *et al.*, 2005), however, *P. aeruginosa* adaptive ability causes difficulties for the sensitivity of these methods, Therefore, it has become necessary to develop genotype-based characterization systems capable of accurately identifying these microorganisms despite any phenotypic modifications, DNA marker allow rapid identification of species, Among DNA marker, the Polymerase Chain Reaction (PCR) is highly sensitive specific and rapid method which vastly improved the detection of *P. aeruginosa* especially when using species-specific primer such as 16SrRNA, *gyrB*, *toxA*, and 16S–23SrRNA genes (Xu *et al.*, 2004). Selective amplification of *Pseudomonas* 16S rRNA gene by PCR has been used to detect differentiate *Pseudomonas* species from clinical and environmental samples (Porteous *et al.*, 2002). It is also used for genus - or species - level identification of *P. aeruginosa* (Spilker *et al.*, 2004). The greatest gains with this method can be made when it is used for the early detection of *P. aeruginosa* (Karpati and Jonasson, 1996). Species specific PCR targeting the outer membrane lipoprotein gene *oprL*, was developed (Lim *et al.*, 1997). It was used for the direct detection and identification of *P. aeruginosa* in clinical samples (Nde *et al.*, 2008). Positive PCR results were obtained using primer specific for *oprL* gene for 150 strains of *P. aeruginosa* isolates, including strains of clinical and environmental origin (Jaffe *et al.*, 2001). The aims of this study are: Isolation and identification of *P. aeruginosa* from clinical sample, using phenotypic test, and polymerase chain reaction using species specific primers.

2. Methodology

2.1. Sampling

Between August and December 2010, 165 samples were taken from three general hospitals of Sulaimani, Erbil and Koya as 55 for each. The swab samples were taken from patients with infected wounds including, burn, wound and ear infections, and urine samples were taken from patients suffering from urinary tract infection (UTI).

2.2. Phenotypic identification of *P. aeruginosa*

According to Chessbrough (1991), the swabs were enriched in brain-heart infusion broth, plated onto MacConkey agar. A single colony was selected and inoculated on the selective medium (cetrimide agar). Then phenotyping characteristics of *P. aeruginosa* was described after Gram staining, including pigments production after incubation at 37°C, and the biochemical test done according to MacFaddin, (2000) which include: motility, Indole

production test. Methyl red test. Voges-proskauer (VP) ,Citrate utilization test , urease activity ,Oxidase test and the catalase test , then API 20E System used for biochemical test which consists of 20 microtubes, containing dehydrated substrates. These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents and the identification is obtained by referring to the analytical profile index.

2.3. Antimicrobial susceptibility test

The most widely used method for susceptibility testing is the disc agar diffusion method which based on Mueller Hinton medium according to Ferraro *et al.* (2000)

2.4. DNA Extraction

Genomic DNA was prepared for twenty four samples chosen according to Cardinal *et al.*, (1997) method with some modification as follow: Ten ml overnight cultures were prepared in broth media from fresh single colony. Cells were harvested in a centrifuge for 5 min at 6000 rpm. then suspended in 200 µl 1xTE buffer (pH8) ,then 30 mg/ml lysozyme to the cell Suspensions was added and incubated for 2 hours at 37°C. After the incubation, 370 µl, 1x TE (pH 8) containing Proteinase K (1mg/ml) was added for 1 hour.then 30 µl, 10% SDS were added. The samples were then incubated for 1 h at 37°C.then phenol Chloroform extraction was performed using one equal volume of phenol /chloroform/isoamyl alcohol (24/24/1) for 30 minutes and then, samples were centrifuged for 5 min at 6000 rpm.then the aqueous phase was transferred into a clean eppendorf tube and the genomic DNA was precipitated by the addition of cold isopropanol (one equal volume) after addition 10% of the volume by ammonium acetate and. Finally, the pellet was dissolved in TE buffer.

2.5. Application of Polymerase Chain Reaction

Twenty four samples of *P. aeruginosa* were selected for analyzing by PCR. The PCR mixture reaction were mixed in a sterile (0.5ml) eppendorf tube including : 2.5 µl of PCR buffer(500 mM-KCl, 100 mM Tris-HCl [pH 8.4]), 1u Taq polymerase, 0.8 µl MgCl₂ (15 mM) and 2.5 µl dNTPs(dGTP, dTTP, dATP, and dCTP; 2 mM each) and 2 µl of Forward and Reverse prime in concentration of 10Pmol, the volume complete up to 25 µl by sterilized double distilled water, the sequence of the primers used are : Pp16S-F GACGGGTGAGTAATGCCTA and Pp16S-R CACTGGTGTTCCCTCCTATA for *P. aeruginosa* .and F. *oprL* ATGGAAATGCTGAAATTCGGC, R. *oprL* CTTCTTCAGCTCGACGCGACG which designed on previously published studies (Spilker *et al.*, 2004, and Deschaght *et al.*, 2009) which amplifies 956 and 504 bp respectively. the amplification program was run as follow: One pre cycle of 95°C for 2 minutes, 30cycles (92°C for 1 minute,55 - 59°C for 1 minute depending on the type of the primer and 72°C for 1 minute)and One final extension cycle of 72°C for 10 minutes, Then the amplified product was running by 1.2% agarose gel electrophoresis which stained using ethidium bromide .

3. Results and Discussion

3.1. Sampling

Out of the 165 specimens that were collected from the three hospitals, 43 isolates successfully were diagnosed as *P. aeruginosa*, representing 26.06% of total isolates and the highest percentage of *P. aeruginosa* was obtained from burn samples (%10.9) whereas the lowest percentage were obtained from Otitis samples (%1.81). This is agreed with R'auf (2003) who recorded highest percentage of *P. aeruginosa* among burn infection followed by wound (41.7%) and ear infection (28%), This is because *P. aeruginosa* is the third most-common pathogen associated with hospital-acquired infections (Moreau-Marquis *et al.*, 2008). *P. aeruginosa* introduced into areas only when devoid of normal defenses example or when membranes and skin are disrupted by direct tissue damage, when intravenous or urinary catheter is used, beside *P. aeruginosa* is more resistant to many antibacterial agents and therefore becomes dominant and important when more susceptible bacteria of normal flora are suppressed.

3.2. Phenotypic test

The result of the staining showed that *P. aeruginosa* produces green pigment and characteristic odour on cetrimide agar. *P. aeruginosa* reacted positively to catalase and oxidase tests, while it was negative for methyl red, Voges Proskauer and indole. It slowly hydrolyzed urea, utilized Simmon's citrate the biochemical properties of the organism recorded in this study are the same as obtained by Abro and his coworkers (2009). In general biochemical methods have, to a certain extent, facilitated the identification of *P. aeruginosa*; however, some of these methods are time consuming. No difference obtained using API 20E system of biochemical tests. Although API system was shown to be practical, fast, however it can be applied for the identification of microorganisms only at the genus level. Many other additional morphological, biochemical, and physiological tests are always needed to obtain the exact identification.

3.3. Antibiotic Sensitivity

The results showed all isolates were completely susceptible to Ciprofloxacin, Imipenin, Lincomycin, Piperacillin and Tobramycin. This result is in agreement with Alsaimary *et al.*, (2010), this is because it is a type of quinolons, which prevents bacterial DNA from unwinding and duplicating by binding to one of several DNA involved in replication such as DNA gyrase (Hooper, 2001). On the other hand, all isolates showed complete resistance to seven antibiotics these include: Cefotaxime, Chloramphenicol, Clindomycin, Erythromycin, Gentamycin, Nitrofurantoin and Pincillin. This is called multiple drugs resistance (MDR). MDR *P. aeruginosa* has been previously reported (Loureiro *et al.*, 2002). This resistance results from the complex interaction of several mechanisms, which tend to inactivate the antibiotics or prevent their intracellular accumulation to inhibitory levels (Hancock and Speert, 2000).

However the isolates differ in their susceptibility to other antibiotic these include: 37(86.04%) of the isolates were sensitive to Amikacin and (6) 13.95% of the isolates were resistant to this antibiotic. For Nalidixic acid 32.5% were sensitive and 67.5% were resistance. And 41.86%were sensitive to Rifampicin and 58.1% were resistant. Whereas 6.9% were sensitive to Tetracycline and 93.1% were resistant, 11.6%isolates were sensitive to Trimethoprim and 38 (88.3%) isolates were resistant

3.4. Genomic DNA isolation

The result showed that, the full amount of DNA obtained(Figure: 1) using this protocol was very efficient method for DNA extraction from *P. aeruginosa*, since good yields of genomic DNA were obtained .

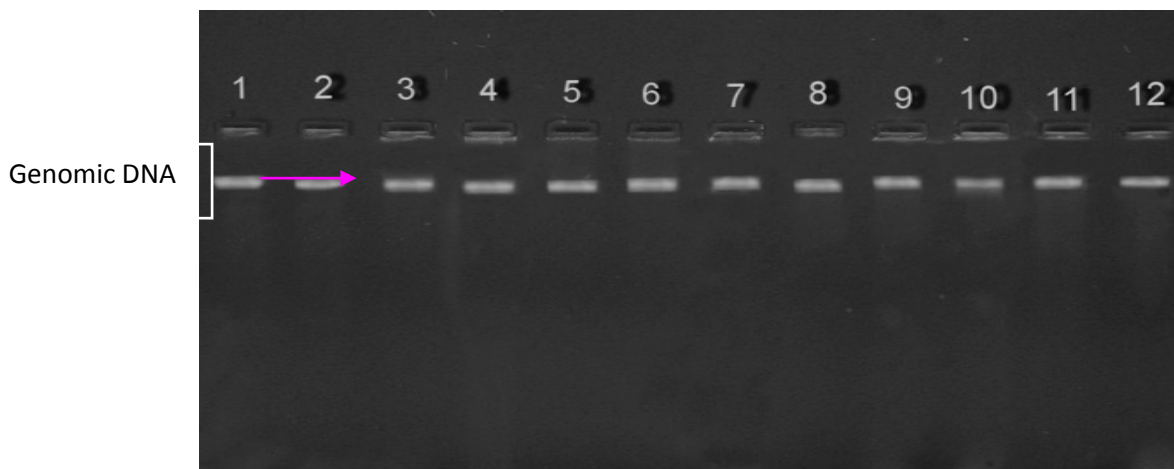


Fig 1: Agarose gel electrophoresis 1% at 80Volt /cm of whole genomic DNA of *P. aeruginosa* isolates of Sulaimania. Lane 1-2-3 from Burn, 4-5 from wound, 6-7 from Urine, 8 from Otitis, 9-10 -11 from Burn of Erbil and 12 from Wound of Erbil.

3.5. PCR Analysis

Two primer pairs were used in this study; the pair PA16S-F and PA16S-R which was specific to *P. aeruginosa*. These primers targeted the variable regions in the 16S rRNA gene. PCR assays employing this primer pair produced DNA products of the predicted size (Figure: 1). 16S rRNA gene sequence offered a useful method for the identification of bacteria. It had long been used as a taxonomic method in determining the phylogenies of bacterial species (Drancourt et al., 2000)

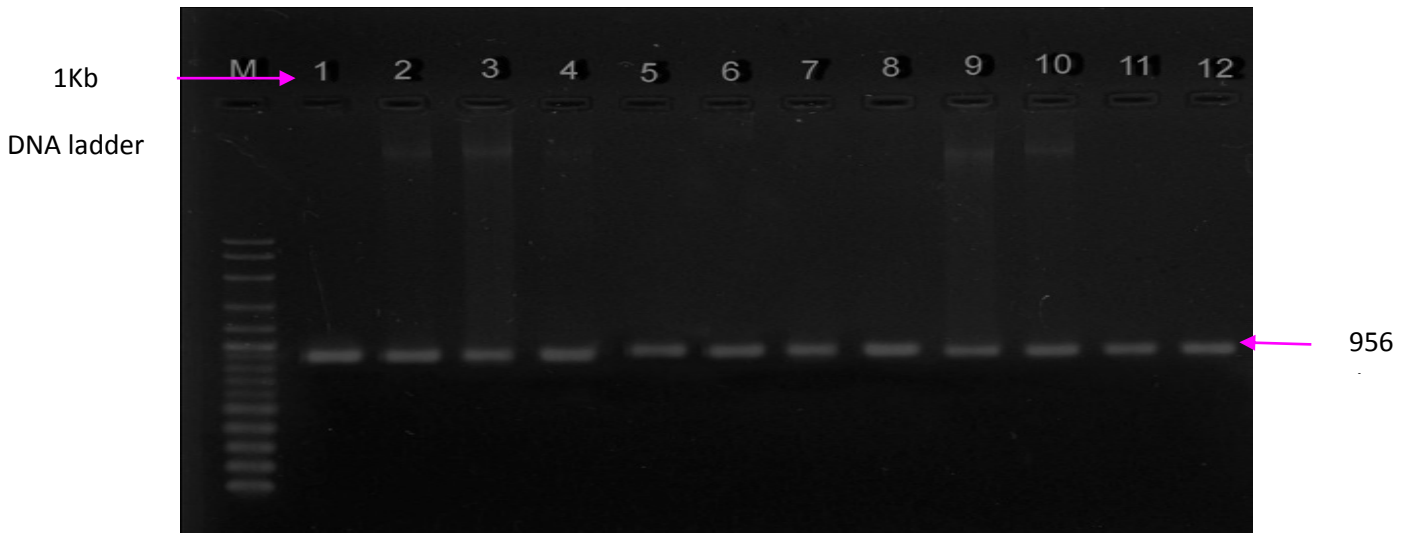


Figure 2: 956 bp PCR products of 16S rDNA which was specific for *P. aeruginosa* were identified in all samples in 1.2% agarose gel electrophoresis. M 1kb DNA ladder 1-12 were various samples of *P. aeruginosa* isolates.

The other pairs of primers used To detect *p. aeruginosa*, were that targeting the *oprL* gene (encodes for membrane lipoprotein), since it has been reported to be conserved in *p. aeruginosa* (Daniel *et al.*, 1997). Using this pair of primers, 504 bp amplified band was obtained which was specific for *oprL* gene in *p. aeruginosa* isolates (Figure:3).

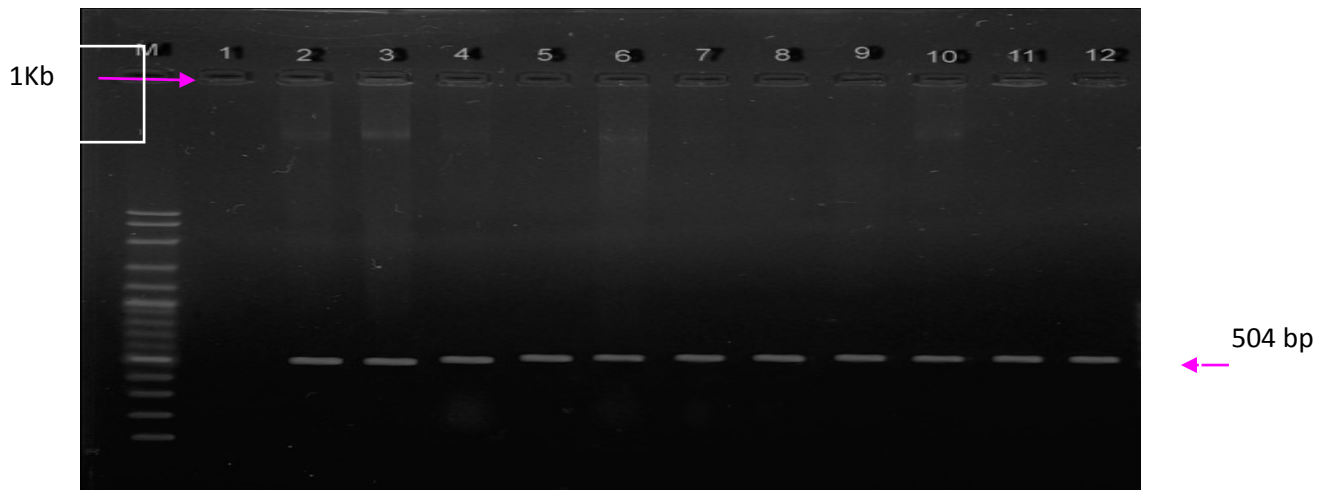


Fig3: 504 bp PCR products of *oprL* DNA were identified in all samples(except one) using 1.2% agarose gel electrophoresis. M 1kb DNA ladder 1- 12 were various samples of *P. aeruginosa* isolates.

The sensitivity for the detection of *p. aeruginosa* was 22/24 (92%) compared to 16SrRNA based PCR which was 100%. the close explain for this is the low sensitivity of *oprL* gene Thus, combined PCR-based detection of these can be performed . Pirnay and his coworkers

(2000) used three kind of PCR to detect and quantify *p. aeruginosa* in wound biopsy samples: conventional PCR, (ELISA)-PCR, and Real-time detection *oprL* gene.

4. Conclusion

As epidemiologic studies on *P. aeruginosa* in patients are hindered by problems related to phenotypic variations of bacterial isolates throughout the period of infection, because phenotypic identification systems do not allow for reliable determination of the number of *P. aeruginosa* strains, whether the patient is infected by different strains over a period of time, or whether the isolated strains differ according to the site where the sample was taken, beside it was time consuming so there is need for qualitative and quantitative tests that are more rapid than bacterial culture such as PCR ,which applied successfully in this study .

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