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PCR Detection of Some Virulence Genes of *Pseudomonas aeruginosa* in Kirkuk city, Iraq

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Abstract:
This study was carried out at Kirkuk hospitals, in Kirkuk, Iraq, during March to July, 2017. Totally 120 swap were collected and cultured (90 patients suffered from burns, and 30 patients suffered from wounds) for the detection of *Pseudomonas aeruginosa*, the isolated bacteria were identified by biochemical tests, API 20 E and Vitak 2 system. Out of the total samples, 32 (26.6%) isolates of *P. aeruginosa* were isolated, distributed as (28) from burns and (4) from wounds. According to gender and age group, the study showed the highest rate of *P. aeruginosa* in the male (56.2%), and in young patients (30 %) between the ages of 23 and 28 years compared to the elderly; The DNA was isolated from isolates of *P. aeruginosa* were extracted by QIAamp DNA mini kit, the concentration for all 32 DNA samples were between 65-100 ng/µl and the purity were between 1.8-2 . Polymerase Chain Reaction was used for detecting the virulence genes (*opr L* and *tox A*). The result showed that 31 (96.8 %) isolates were positive for *opr L* and 32 (100%) for *tox A* genes.

INTRODUCTION:

Pseudomonas aeruginosa was ranked as the most frequent pathogen in surgery [1] Opportunistic diseases brought about by *P. aeruginosa* are a genuine medicinal issue, and quinolone resistant *P. aeruginosa* could be recovered from clinical cases [2]. It is most harmful to individuals whose immune systems have been compromised similar to those with in AIDS, cancer, burns, cystic fibrosis, and neutropenia. Several infections can be acquired in the hospital such as wound, burn, urinary tract, and eye and outer ear infections, as well as meningitis and necrotizing pneumonia [3]. The diversity of *P. aeruginosa* strains has been frequently investigated through molecular typing methods, including ribotyping, repetitive-element-based PCR (rep- PCR), arbitrarily primed PCR (AP-PCR), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphic (RFLP) DNA analysis, random amplified polymorphic DNA (RAPD) assay, and pulsed-field gel electrophoresis (PFGE) [4].

PCR has the potential for identifying microbial species rapidly by amplification of sequences unique to a particular organism [5]. L and I lipoproteins are two outer membrane proteins of *P. aeruginosa* responsible for inherent resistance of *P. aeruginosa* to antibiotics and antiseptics. As these proteins are found only in this organism, they could be a reliable factor for rapid identification of *P. aeruginosa* in clinical samples [6]. The pathogenesis of *Pseudomonas aeruginosa* opportunistic infections is multifactorial, as suggested by the large number of cell-associated and extracellular virulence determinants; some of these determinants help colonization, whereas others facilitate bacterial invasion. The virulence of *P.* aeruginosa depends mainly on two types of virulence determinants: (i) virulence factors involved in acute infection, they being usually secreted and membrane bound factors. *P. aeruginosa* considered have a large number of virulence factors suchas exoenzyme S ,exotoxin A, , elastase and sialidasean [7] addition to several of the other extra cellular products. Exotoxin A encoded by the *toxA* gene which has the ability to inhibit protein biosynthesis just like diphtheria toxin[8][9].

The aim of this study was to isolate *P. aeruginosa* and detect *opr*L and *tox*A virulence genes.

MATERIALS AND METHODS:

Bacterial strains collection and identification test:

Totally 120 swap were collected (90 patients suffered from burns, and 30 patients suffered from wounds), of hospitalized burns and wounds patients from Kirkuk hospitals, in Kirkuk, Iraq. during March to July, 2017. All samples were cultured on MacConkey agar, Blood agar, Cetrimide agar, King A and king B medium. The biochemical tests were performed for confirmed the identification the *P. aeruginosa* isolates by oxidase, catalase, motility, IMVIC tests [10]. The biochemical tests result of final identification of *p.aeruginosa* was dependent on Api 20 E, and Vitak 2 systems.

Bacterial DNA extraction and PCR Method:

DNA Extraction:

Bacterial genomic DNAs were extracted with the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's protocols and examined by Electrophoresis apparatus in a 1% agarose extracted DNA then stained with ethidium bromide, and take a look under UV transilluminator.

Nanodrop: DNA was estimated by nanodrop device at 260/280nm, and then preserved at (-20˚C) until used for polymerase chain reaction (PCR) tests.

PCR analysis:

PCR technique was performed for virulence factors genes (exotoxin A (*toxA*) and outer membrane protein (*oprL*) gene in *Pseudomonas aeruginosa* based using specific primers.

PCR amplification was carried out using thermal cycler (BioRad, USA) with specific primers for *oprL* and *toxA* genes. Table (1)

Table (1): primers and their sequence and amplicon.

Amplified gene (Primers)	Sequence (5→3)	Amplicon
oprL	F, 5'-ATG GAA ATG CTG AAA TTC GGC-3' R, 5'-CTT CTT CAG CTC GAC GCG ACG-3'	500 bp
toxA	F, 5' GGT AAC CAG CTC AGC CAC AT 3' R, 5' TGA TGT CCA GGT CAT GCT TC 3'	352bp

PCR was carried out in 50 µl volume reaction mixtures containing 1 µl of each primer, 10 µl of crude template DNA and 25 µl Qiagen master mix. The annealing temperature was 55°C for, *oprL* and *toxA*. [11].

PCR product analysis: has examined by Electrophoresis apparatus in a 1% agarose substance by using buffer, then stained with ethidium bromide, and take a look under UV transilluminator.

Results: In current study, From 120 specimens 32 (26.6 %) isolates of *Pseudomonas aeruginosa* (28 (31%) from burns and 4 (13%) from wounds) were shown in the following table (2).

Table 2. number and percentage of *Pseudomonas aeruginosa*.

Type of samples	Total samples	Positives isolates	Negatives isolates	Percent %
Burns	90	28	62	31%
Wounds	30	4	26	13%

A total of %43.2 (n=14) and %56.2 (n=18) of *Pseudomonas aeruginosa* strains were isolated from Female and Male patients, respectively, table (3).

Table (3):Distribution and percentages of *P. aeruginosa* according to gender.

	Male	Female
Positive sample	18	14
Percentage %	56.2	43.7

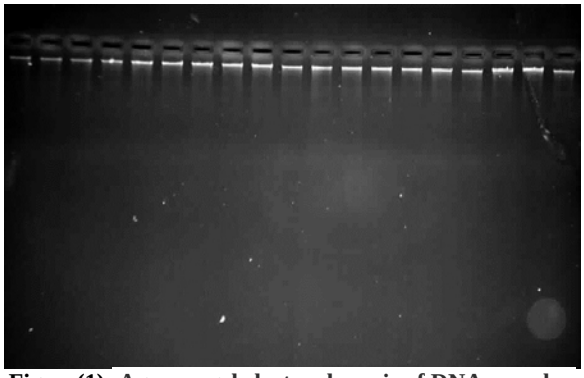
Also, the patients ages ranged between 5 to 64 years, the majority between 23-28 years .table (4).

Table (4): Frequency (%) of the Burn and wound Patients Involved in *P. aeruginosa* According Different Age Groups.

Age groups(years)	5-10	11-16	17-22	23-28	29-34	35-40	41-46	47-52	53-58	59-64
Rate(%)	11	15	22	30	5.6	6.4	0	5.5	4.5	0

The result revealed that the concentration of all DNA samples of the thirty two *Pseudomonas aeruginosa* isolates were between 65-100 ng/ul and the purity was between 1.8-2, figure (1).

Results showed the distribution of virulence genes in *Pseudomonas aeruginosa* which are 31 (96.8 %) isolates were positive for *oprL* and 1 (3.1%) were PCR negative while the *toxA* gene were detected in all of the 32 (100%) *Pseudomonas aeruginosa* isolates collected, table (5).



Figure(1): Agarose gel electrophoresis of DNA samples.

Table (5): number and percentage of genes of positive isolates.

Type of genes	Positive isolates	Percentage
oprL	31	96.8 %
toxA	32	100%

For detection of virulence genes of *Pseudomonas aeruginosa* (*oprL* and *toxA*) PCR reactions were done and the following results were obtained. PCR results of *oprL* gene (500bp) and *toxA* gene (352bp) expression are shown in Figure 2 and 3 respectively.

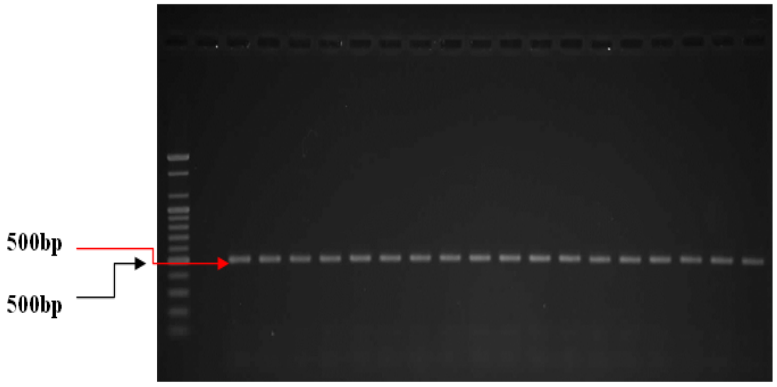


Figure (2): Agarose Gel electrophoresis of PCR product for the detection of *oprL* gene (504bp) using 1%agarose for 90 min at 70 volt , stained with ethidium bromide, M: 100bp DNA Ladder, Lanes 1 negative control. Lanes (2-18):Positive for *oprL* gene (504bp).

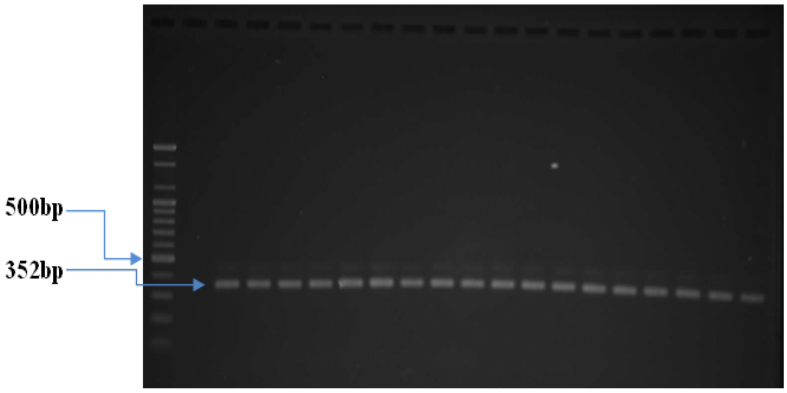


Figure (3): Agarose Gel electrophoresis of PCR product for the detection of *toxA* gene (352bp) using 1%agarose for 90 min at 70 volt , stained with ethidium bromide, M: 100bp DNA Ladder, Lanes 1 negative control. Lanes (2-18):Positive for *toxA* gene (352bp).

DISCUSSION:

Pseudomonas aeruginosa is a leading cause of nosocomial infections. Infections caused by it are often severe and life threatening and difficult to treat because the organism is inherently resistant to many drug classes (MDR) and is able to acquire resistance to all effective antimicrobial drugs. Over the years, P. aeruginosa contributes substantially to morbidity and mortality related to surgical site infection (SSI) worldwide, the third most commonly reported nosocomial infection.[12] also, Burn patients are more liable to get infections in comparison with other patients because of their damaged skin barrier and suppressed immune system, in addition to extended hospital stay and invasive therapeutic and diagnostic procedures [13].The findings come with matching results almost with others in Karbala city, Iraq [14], which showed that the highest percentage of isolated bacterial in burn patients had the bacteria *pseudomonas aeruginosa* , (45%) and (49%) with Alhamdy [15]. Many burn patients die as a result of infection during their hospital courses. The rate of infection in burn cases is extremely high in developing countries. [16][17] . This may be due to the pervalence of low level socioeconomic groups of patients in whom poor hygienic conditions prevail [18]. Also disparity in prevalence rate among several studies may be attributed to differences in hygienic practices and geographical location. According to gender and age group, the result of this study shows the rate of *P. aeruginosa* in the male (56.2 %), and 30% in the young patients (ages 23 to 28 years) compared to the elderly, agree with [19] indicates that males in this age group are more active and involve in different clinical hygiene practices, even in hospital environment.. This result is comparable with the study of Okon et al in Nigeria , which recorded that male patients showed a record of 52.8% and the highest frequency of this bacterium (20.7%) was found in age group of 29 years and below [20]. On the other hand, these results disagree with results in Karbala city, Iraq [14], studies of Shewatatek et al. [21] in Ethiopia and Ekrem and R okan in Al-Sulaimania city, Iraq, where results of the studies showed higher occurrence of the bacterium in female and elderly patients [22].

Pseudomonas aeruginosa produces many of virulence factors whose expression is arranged by different systems [23]. A recent studies reveal *P. aeruginosa* is most frequent pathogen that formed many of virulence factors example *ToxA, exoA* , *oprL* and *oprI* genes [24] .

PCR results showed that, 31 of 32 *P. aeruginosa* isolates was positive for the *oprL* gene with amplified size (500 bp) in a percentage (98%), Similarly in this study, all of the isolates (100%) were remarkably positive for both *oprI* and *oprL* genes [25]. *P. aeruginosa* possesses a variety of virulence factors that may contribute to its pathogenicity. Our results showed that *toxA* gene were detected in all 32 tested strains of *P. aeruginosa* , The differences in the distributions of virulence factor genes in the populations strengthen the probability that some *P. aeruginosa* strains are better adapted to the specific conditions found in specific infectious sites [26] that may returned to the different environmental and geographical sources. the prevalence of *pseudomonas areugenosa* and percentage of virulence factors genes depend on several causes including nature of places, immune status of patients, degree of contamination and type and virulence of strain [27].

CONCLUSION:

The outcome conclusions of this study show that high rate of infected wounds and burns of *P. aeruginosa* probably occur as a result of wide use and abuse of antibiotics. Therefore the result of this study may be as a recommendation to the correct use of antibiotics in treatment of patients. PCR seems that simultaneous use of specific primers different virulence factors genes as (*oprL* and *toxA*) of *P. aeruginosa* provides more confident detection of *P. aeruginosa*. Also its differences in the distributions of virulence factor genes in the isolated strains need further studies for finding out the actual role of these genes of *P. aeruginosa* from different sources. PCR showed that all *P. aeruginosa* strains do not necessarily have similar virulence genes.

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