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RESEARCH PAPER

Evaluating the prevalence of virulence factor gene and biofilm production in *Pseudomonas aeruginosa* isolated from different clinical samples.

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A B S T R A C T:
Pseudomonas aeruginosa is considered a resourceful pathogen; which has several essential virulence effectors such as exoenzyme, exotoxin and biofilm might help to it is infection. This study aimed to investigate the frequency of exoS gene, the determination of biofilm production and antimicrobial resistance among clinical samples of *P. aeruginosa*. In our study, 227 specimens of *P. aeruginosa* collected from different clinical specimens which were attending public hospitals in Erbil city. Antimicrobial resistance of samples identified by Kirby-Bauer disk diffusion method. Through PCR virulence gene exoS was studied. Biofilm production measured by both Congo Red Agar (CRA) and tissue culture plate method (TCP). Among 227 clinical samples, 40 (17.6%) were positive for *P. aeruginosa*. Imipenem was showed most effective antibiotic 95% against *P. aeruginosa*. Incidence of exoS gene was 70% within the *P. aeruginosa* isolates. Moreover, around 75% of clinical samples produced biofilm and approximately 40% of them produced strong biofilm. Our study showed that the incidence of bla exoS gene and biofilm formation, which are common virulence factors in the clinical samples, especially in burn patients, and are a severe problem in the treatment of the patient.

KEY WORDS: *Pseudomonas aeruginosa*, *Bla* exoS gene, Antibiotic resistance, Biofilm
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1.INTRODUCTION :

Pseudomonas aeruginosa has developed as an important of nosocomial infection. It causes infection especially in the immunocompromised hosts as seen in the patients with severe diabetes, burns and chronic renal disease also individuals with HIV infection (Candel *et al.*, 2019, Wang *et al.*, 2019, Kang *et al.*, 2019, Azeez and Bakr, 2019).

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It causes different types of infection such as septicemia, pneumonia, burn wounds, otitis and keratitis (Kang *et al*., 2019, Aka, 2015). This organism is often resistant to a large number of antibiotics and enters the blood, causing septicemia (Minasyan, 2019). The production of toxins, enzymes and also biofilm formation may contribute to its pathogenicity (Aka and Haji, 2015, Hamad, 2018, Ahmed and Salih, 2019). When a bacteria formed a biofilm, it promotes growth and survival, it becomes extremely difficult to be destroyed (Georgescu *et al*., 2016). Biofilm supports the colonization of bacteria, which is showing resistant to most antibiotics (Yin *et al*., 2019). Among bacterial pathogens, *P. aeruginosa* inherent resistance to antibiotics such as aminoglycosides, carbapenems, penicillins,

quinolones and cephalosporins. Furthermore, it has been known to obtain unusual resistance genes via transformation, transduction or conjugation (Amirmozafari *et al*., 2016, Pobiega *et al*., 2015). Besides, there is a major virulence factor which was known by the type III secretion system (T3SS). It has a vital function to transfer effector proteins into the host. *Pseudomonas aeruginosa* produces irresistible proteins such as exoS and ExoU (Horna *et al*., 2019). ExoS is the main cytotoxin that is essential for colonisation, invasion and bacterial spreading during infection (Ruffin and Brochiero, 2019).

The vital virulence elements of *P. aeruginosa* is the biofilm which is leading to colonisation of microorganisms, and it plays against antimicrobial agents (Verderosa *et al*., 2019). The primary purpose behind of our study was to investigate the frequency of exoS gene, the determination of biofilm production and antimicrobial resistance among clinical samples of *P. aeruginosa* in Erbil north of Iraq.

1. MATERIALS AND METHODS

1.1.Bacterial isolates and identification test

Our samples were collected from different clinical specimens such as wounds, respiratory tract, urine, blood, burn and sputum. Each isolate was cultured by using different agars such as MacConkey agar and Blood agar then incubated at 37°C overnight. Identification was made by using biochemical tests (Jabalameli *et al*., 2012). All bacterial isolates were stored in a microtube containing tryptic soy broth with glycerol 20% and stored at −70°C until further investigation (Magalhães *et al*., 2016).

1.2.Methods:

Study population and specimens

In our study, 227 clinical samples were collected from different clinical specimens which were attending public hospitals in Erbil city from September 2017 to March 2018. Antimicrobial resistance of samples was identified. Through PCR virulence gene exoS was studied. Biofilm production was measured by both Congo Red Agar (CRA) and tissue culture plate method (TCP).

Antibiotic susceptibility tests

Antibiotic susceptibility test was used through the Kirby-Bauer disk diffusion method (Merck, Germany) agar according to the guideline of Clinical and Laboratory Standards (Magiorakos *et al*., 2012): Penicillin (P 10ug), Rifampicin (R 5ug), Bacitracin (Baci 10ug), Ciprofloxacin (Cip 5µg), Amikacin (Am 10ug), Azithromycin (Azi 10 ug), Cefotaxime (Cef 30 ug), Ceftriaxone (Cef 30ug), Meropenem (Mer 10 ug) and Imipenem (Imi 10ug).

Identification of bacterial isolates as *P. aeruginosa* by PCR:

To know the bacterial isolates as *P. aeruginosa*, all samples were checked for detection of exoS gene with specific primers as explained in Table 1.

PCR based genotyping assay

Genomic DNA was isolated from bacterial cells harvested from overnight culture according to the previously described method (Smet *et al*., 2009). The extraction kit was provided by (GeNet Bio, Korea). PCR amplification for exoS gene was performed according to the manufacturer program for amplification of target gene: initial denaturation 94°C for 5 min; one cycle and denaturation step 94°C for 30 seconds; annealing step 58°C for 30 seconds; amplification step 72°C for 30 seconds; and the final amplification step was 72°C for 5 minutes. To investigate the PCR products, 5µl of PCR product run on 1% agarose gel in trice boric acid EDTA buffer, after electrophoresis, visualised under ultraviolet transilluminator.

Table 1: Nucleotide sequences used as a primer for PCR amplification.

Name of the gene	Name of primers	Nucleotide sequences start from (5'-3')	PCR product size (bp)	Temperature for annealing	Ref.
Bla exoS	*Bla* exoS forward	ATGTCAGCGG GATAT CGA AC	230	58^{o}C	(Georgescu *et al*., 2016)
	Bla exoS reverse	CAG GCG TAC ATC CTG TTC CT			

1.3.Detection of biofilm formation

Tissue culture plate method

Bacterial colonies were grown for 24hrs at 37°C in Trypticase Soy Broth (Merck Darmstadt, Germany). Then, dilution was done in TSB medium and only 150 µl of dilution transferred for

sterile microtiter plates. Next, incubated for 24 hrs at 37°C without shaking, then washed by phosphate buffer saline three times. Later, 100 µl of methanol was added and incubated for 15 minutes. After removing the solution the plate was dried at room temperature.

The next step was adding crystal violet for 20 minutes, and the bound dye was released by adding acetic acid. The reading of optical density (OD) was done through ELISA reader (Biotek elx800). The assays were repeated for three times. Un-inoculated medium was used in order to determine background OD as a control.

According to the ELISA reader results, the samples were grouped into four different groups based on the reading: non-biofilm produce group (OD test <ODc), weak biofilm produce group (ODc< OD < 2× ODc), moderate biofilm produce group (2× ODc< OD < 4X ODc) and strong biofilm produce group (4× ODc< OD) (21, 22).

Congo red agar method

Bacterial suspension inoculated into a prepared solid medium (BHI) added with 5% sucrose and Congo red. It was made as a concentrated solution and sterilised by autoclave for 15 minutes at 121˚C, then added after cooling the agar to 55˚C. The agar plate was inoculated and incubated for 24-48 hours at 37˚C. The black colonies plus a dry crystalline were positives in contrast, the dark colonies without a dry crystalline were pointed as negatives, and weak producers remained as a pink. The assays were repeated for three times.

Table 2: Distribution of P. aeruginosa in different clinical samples

Results	Urine	Bronchial wash	Sputum	wound	Burn	Total	p-value
positive	7 (3.1%)	4 (1.8%)	6 (2.6%)	9 (4.0%)	14 (6.2%)	40 (17.6 %)	<0.001
Negative	113 (49.8%)	18 (7.9%)	12(5.3%)	37(16. 3%)	7(3.1%)	187(82.4 %)	
Total	120 (52.9%)	22 (9.7%)	18 (7.9%)	46 (20.3 %)	21 (9.3%)	227(100. 0%)	

Table 3: Screening of *P. aeruginosa* isolates for detection of biofilm formation by tissue culture plate and Congo Red Agar (CRA) methods.

Biofilm formation	Tissue culture plate	Congo red agar
Strong	14(35%)	18(45%)
Moderate	16(40%)	10(25%)
Weak	9(23%)	6(15%)
None	1 (2%)	6(15%)
Total	40	40

Table 4: PCR-based genotyping assay

PCR results	Source of samples					Total
	Urine	Bronchial wash	Sputum	wound	Burn	
Virulence gene exoS (+)	6 (15%)	2 (5%)	4 (10%)	6 (15%)	10 (25%)	28 (70%)
Virulence gene exoS (-)	1 (2.5%)	2 (5%)	2 (5%)	3 (7.5%)	4 (10%)	12 (30%)
Total	7 (17.5 %)	4 (10%)	6 (15%)	9 (22.5%)	14 (35%)	40(100.0%)

2.8. Statistical Analysis

Statistical analysis including the chi-square test with P values less than 0.05 were considered as significant. Statistical program for social sciences (SPSS V.23) was used for the analysis of the results.

2. RESULTS AND DISCUSSION

From 227 samples, 40 (17.6%) were positive for *P. aeruginosa* (Table 2). Also, the rate of isolates produced biofilms 35%, 40% respectively strong and moderate biofilms. In contrast, the ratio of weak or non- biofilm formation were about 25% (Table 3). The highest level of resistance was observed to penicillin, rifampicin, and bacitracin 100%, followed by ciprofloxacin and amikacin 92.5, 87.5% respectively, while the least resistance exhibited to imipenem 5%. Also, the level of resistance to cefotaxime and meropenem 82.5%, 75% respectively. Furthermore, the sensitivity exhibited to ciprofloxacin, azithromycin and ceftriaxone 80% (Figure 1). Figure 2 illustrated the amplification for the detection of the exoS gene. The incidence of antimicrobial resistance within the *P. aeruginosa* isolates was (70%). Moreover, it was found the highest frequency exoS gene 25% in burn infection (Table 4).

DISCUSSION:

Pseudomonas aeruginosa is an important pathogen of hospital-acquired infections, and it has resistance to a wide variety of antibiotics in clinical samples, particularly in (burn, UTI, and wound) infections (Azimi *et al*., 2016).

According to the results, the association among different clinical samples was highly significant (P<0.001). The high frequencies of *P. aeruginosa* in various clinical samples have been earlier informed (Aka and Haji, 2015).

The peak level of resistance was observed to penicillin, rifampicin, vancomycin and bacitracin 100%, and it was showed low resistance 5% to imipenem. Earlier resistant to imipenem in Erbil was stated 20% and 4% (Azeez and Bakr, 2019) followed by ciprofloxacin and amikacin 92.5%, 87.5% respectively, Similar results have been reported by (Azeez and Bakr, 2019, Azimi *et al*., 2018). Also, the level of resistance to cefotaxime and meropenem were 82.5% and 75% respectively. Furthermore, the sensitivity exhibited to azithromycin and ceftriaxone was 80%.This wide resistance showed by *P. aeruginosa* against most effective antibiotics might be related to misuse or overuse of antibiotics (Pachori *et al*., 2019).

Biofilm formation has been measured as a prime virulence factor of pathogenicity (Tharwat *et al*., 2017). The current study identified that (98%, 85%) of the isolated samples produced biofilm by TCP and CRP method respectively, this is agreed with (Jabalameli *et al*., 2012, Hamad, 2018).

The biofilm production facilitates to ascent bacterial resistant strains and eases the efficacy of antibiotic therapy (Bahador *et al*., 2019). ExoS is a major cytotoxin that is required for bacterial colonisation and invasion. In addition, it needs for bacterial dissemination during infection (Yousefi-Avarvand *et al*., 2015). Our results identified exoS gene most prevalent (70%) within the *P. aeruginosa* isolates which in agreement with other study and similar to (Bahador *et al*., 2019, Mingxiang *et al*., 2018). In Poland, Pobiega revealed a rate for exoS (92.3%) among clinical samples (Amirmozafari *et al*., 2016).

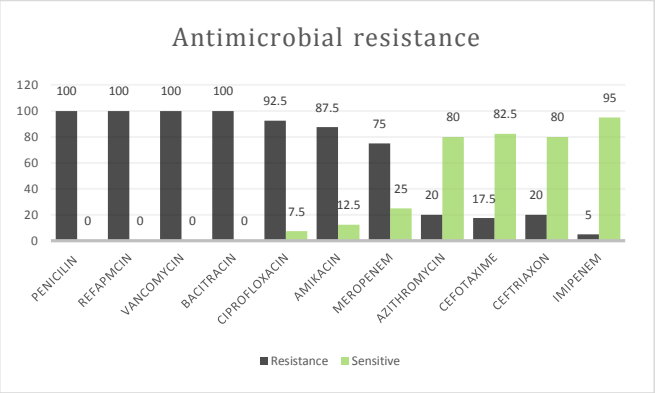

Figure 1: Antimicrobial resistance properties in *Pseudomonas aeruginosa* isolated from clinical infections.

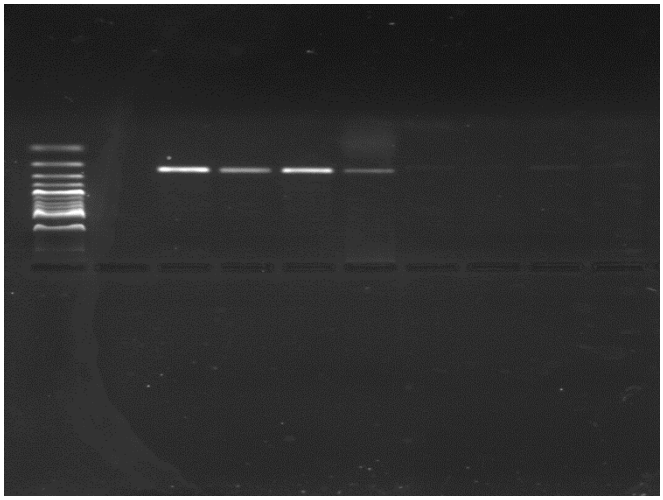

Figure 2: Gel Electrophoresis of the Polymerase Chain Reaction Products Using exoS Gene-Specific Primers. Confirmatory PCR screening analysis of the presence of exoS gene among *P. aeruginosa* clinical isolates. Lane M is DNA ladder (BioLabs); Lane 1 negative control, Lanes 2, 3, 4,5 amplified product of exo S gene (230 bp); Lane 6,7, 8, 9 Negative samples.

3. CONCLUSIONS

In conclusion, our investigation showed that the exoenzyme S is commonly disseminated among the *P. aeruginosa* samples in governmental hospitals. Moreover, a high rate of biofilm producer samples was identified in burn samples, which is a challenge in the treatment of burn patients. Additional studies are needed on other virulence factors to attain more information regarding the antibiotic resistance of *P. aeruginosa*, to find the best way to treat patients more quickly.

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