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Investigation of the relationship between multi drug resistant and *ExoU, ExoS* encoding gene of wound isolated *Pseudomonas aeruginosa*

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Abstract

A total of 20 wounds infections *Pseudomonas aeroginosa* were isolated. *P. aeruginosa* were distributed against antibiotic used. Results showed that 100 % of the samples are resistant to tetracycline, 56% to carbenicillin,15 % to aztreonam,5 % to piperacillin/tazobactam,98 % to ceftazidime, and 13% to imipenem. Multidrug resistance was also detected with 60% being resistant to all the 10 antibiotics tested, 35% are resistant to only 4-6 antibiotics, and 5% resistant to all the 3 tested antibiotics. The biofilm studded showed 14 isolates among 20 total isolates have ability to produced biofilm, 50% Of biofilm isolates belong to group C, which indicated MDR , these results focusing upon the relationship between MDR and biofilm producing. The results showed that 16 isolates of the 20 *P. aeruginosa* clinical isolates harbored only *ExoU* toxin gene, another 4 isolates harbored only the *ExoS* toxin gene, only one isolate carry both toxin genes, while no isolates have none. This indicates that the presence of virulence factors, such as the toxin genes, enhances the tolerance of *P. aeruginosa* in the host, and thus necessitates extensive antimicrobial treatment. Therefore, antibiotic resistance develops due to selective pressure exerted by the toxins produced.

Keywords: PCR, *ExoU, ExoS*, MDR.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that is a common cause of hospital-acquired infections, particularly infecting patients with predisposing factors, such as burn victim, immunocompromised hosts, or those with metabolic disorders. In cystic fibrosis (CF) patients, *P. aeruginosa* is believed to be a major contributory factor to chronic lung infections, which could form biofilm and adhere to human mucin in the lower respiratory tract (Whiteley *et al.*, 2001). The organism can only be eradicated in the early stage of colonization, while reduction of bacterial density is desirable during chronic colonization or exacerbations. It is not surprising that *P. aeruginosa* infections are associated with significant morbidity and mortality due to the organism's capacity to adapt easily to changes in the environment, to rapidly develop resistance to antibiotics, and to produce a variety of virulence factors (Mitov *et al.*, 2010). Pseudomonas aeruginosa harbors at least one or more *ExoS, ExoT, ExoU*, and *ExoY* genes that are translated into protein products related to type III secretion systems (TTSS). These products have been demonstrated to show a cytotoxic effect *In vitro*. Furthermore, in clinical studies, the presence of these toxins is associated with a dissatisfactory clinical outcome among patients with *P. aeruginosa* infection. These TTSS-related toxin genes show heterogeneity in terms of their presence in the genome of individual *P. aeruginosa* strain, for example, the presence of lence of type 3 secretion toxins- encoding genes are mutually exclusive (Gendin *et al.*, 2012).

The study was done to determine the prevalence of *ExoU* and *ExoS* type 3 secretion toxins encoding genes and there correlation with multi drug resistance *P. aeruginosa*

Materials and Methods

Isolation and Identification: A total of 20 *Pseudomonas aeruginosa* from wound infection attending in and outpatient, from Baghdad Educational Hospital and some private laboratory. Wound samples swaps were processed as standard protocol. Non lactose fermenting colonies on MacConkey's agar (MA) were processed and identified as *Pseudomonas aeruginosa* by standard biochemical tests (Mac Faddin, 2000) and confirmatory by api20 kit.

Detection of Susceptibility to Antibacterial Agents:

Susceptibility of all the isolates to different antibiotics was determined by the disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (2009). The antibiotic discs used in this study were amoxicillin/clavulanic acid (30mg; 20:10), piperacillin/tazobactam (30μg; 20:10), Cefoxitin (30μg), carbenicillin (30μg), Cefotazidime, (30μg), ciprofloxacin (5μg), Cefepime (30μg), Imipenem (10μg), tetracyclin (5μg), aztreonam (30μg). Each antibiotic concentration was applied on the surface of Muller-Hinton agar plates inoculated with *E. coli* isolates and incubated at 37°C for 24hrs. (Morata *et al.,* 2012).

Haemolysin production: Plate hemolysis test was done for the detection of β-haemolysin produced by *P. aeruginosa.* The bacteria were inoculated onto 5% human blood agar and incubated over night at 37°C (Khalifa *et al.,* 2011).

Congo red test: Pantanella *et al.* (2013)have described method for screening of biofilm formation. Plates were inoculated and incubated aerobically for 24 - 48hrs. at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result

Preparation of bacterial DNA: The DNA to be amplified was extracted from whole organisms by boiling method. The bacteria were harvested from 1.5 ml of an overnight Luria-Bertani broth culture, suspended in sterile distilled water, and incubated at 95°C for 10min. Following centrifugation of the lysate, the supernatant was stored at -20°C as a template DNA stock (Lemarchand *et al.,* 2005).

Uniplex PCR amplification procedure: Detection of *ExoU and ExoS* genes was performed by amplifying the genes by multiplex PCR. The primers sequences were previously reported by (Mitov *et al.*, 2010) and obtained from alpha DNA company (USA). Descriptions and sequences of the PCR primers used in this study are displayed in Table (1). Amplification was performed in a thermal cycler (Eppendorf, Germany) according to the methods described by (Strateva, 2008). The program, for *ExoU* genes the reactions mixtures included an initial denaturation at 94°C for 1min. consisted of 30 cycles of 94°C for one min, specific annealing temperature 72°C for 30sec. and a final extension at 72°C for 90sec. and for *ExoS* the reactions mixtures included an initial denaturation at 94°C for 5min consisted of 30 cycles of 94°C for one min., specific annealing temperature 68°C for one min. and 72°C for 30 sec. and a final extension at 72°C for seven min. in a thermal cycler (Mitov *et al.*, 2010). The detection of multiplex PCR products was performed on 0.8 to 1% agarose gels by electrophoresis and visualized under UV light.

Table (1): Sequence and molecular size of PCR products of *ExoU* and *ExoS* genes.

Gene		Sequence of forward and reverse Primer(5'- 3')	Product bp
ExoU	F	GGG AAT ACT TTC CGG GAA GTT	428
	R	CGA TCT CGC TGC TAA TGT GTT	
ExoS	F	CTT GAA GGG ACT CGA CAA GG	
	R	TTC AGG TCC GCG TAG TGA AT	504

Result and Discussion

In this study, 20 isolates of *Pseudomonas aeroginosa* were isolated from Baghdad Educational Hospital and some private laboratory. The source of these isolates were from wounds infections. The 20 collected isolates were initially diagnosed as *Pseudomonas* to confirm this diagnosis the bacterial isolates cultured on MacConkey agar, blood agar, and Pseudomonas agar under aerobic conditions followed by other diagnostic tests; poitive result for oxidase, catalase, while urease test was negative. Last indentation for the isolates was dependent on the results of api20E. The api system is a standardized and of conventional procedures for the identification of Enterobacteriaceae and other gram negative bacteria (Mac Faddin 2000) this system consider as rapid and easy methods for identification of the bacteria. By using this system, it was confirmed that these isolates were *P. aeruginosa*.

The 20 clinical isolates of *P. aeruginosa* were distributed against antibiotic used. Results showed that 100% of the samples are resistant to tetracycline, 56% to carbenicillin, 15% to aztreonam, 5% to piperacillin/ tazobactam, 98% to ceftazidime, and 13% to imipenem. This increased tolerance to tetracycline is explained by low permeability of bacterial outer membrane (Dubois

et al., 2008). Adwan et *al.* (2009) showed that efflux system is an effective resistance mechanism of most antibiotics. Selective pressure and multiple mutations may up-regulate multidrug resistance systems in *P. aeruginosa* (Alonso *et al.*, 1999).

Table (2) the percentage of isolates belonging to each groups. Multidrug resistance was also detected with 60% being resistant to all the 10 antibiotics tested, 35% are resistant to only 4-6 antibiotics, and 5% resistant to all the 3 tested antibiotics.

Table (2): The multidrug resistance phenotype in *P. aeruginosa*

Groups	Number of antibiotics which resisted by isolates	Numbers of the multidrug resistance isolates	Percentage of multidrug resistance (%)
Group A	1- 3	1	5
Group B	4- 6	7	35
Group C	7- 10	12	60

The biofilm studded showed 14 isolates among 20 total isolates have ability to produced biofilm, 50% of biofilm isolates belong to group C, which indicated MDR, these results focusing upon the relationship between MDR and biofilm producing. Many studies have sought a relationship between multidrug resistance and *P. aeruginosa* biofilm formation. The presence of alginate in *Pseudomonas* biofilms enhances the multidrug resistance by binding and inactivating the antimicrobials and acting at the same time as an impermeable barrier (Hoiby *et al.*, 2001; Drenkard and Ausubel, 2002).

The results showed that 16 isolates of the 20 *P. aeruginosa* clinical isolates harbored only *ExoU* toxin gene, another 4 isolates harbored only the *ExoS* toxin gene, only one isolate carry both toxin genes, while no isolates have none. One hundred percent of the isolates that harbored *ExoS* toxin gene were resistant to 7-10 of the antimicrobials tested. However, the isolates that carry the *ExoU* toxin gene were resistant to 7-10 antibiotics, (Table 2). *ExoU* protein are able to evade host immune response at the beginning of the infection period while those producing *ExoS* protein can decrease DNA synthesis (Wong-Beringer *et al.*, 2008). This indicates that the presence of virulence factors, such as the toxin genes, enhances the tolerance of *P. aeruginosa* in the host, and thus necessitates extensive antimicrobial treatment. Therefore, antibiotic resistance develops due to selective pressure exerted by the toxins produced.

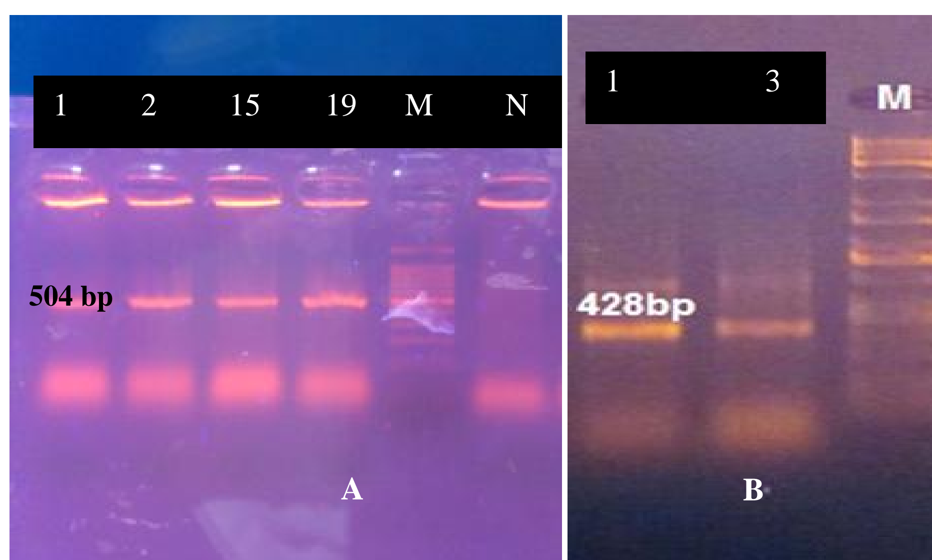

Figure (1): PCR: Agarose gel electrophoresis (1% agarose, 7 v/cm^2) and Ethidium bromide staining. A: detect *ExoS* genes size products (504bp) B: detect *ExoU* genes size products (bands 428bp). Using template DNA prepared by boiling method. Lane M, molecular size DNA ladder (123bp DNA Ladder). Lane N, Negative control.

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