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Bacteriological and Molecular Study for Detection of Virulence Genes in *Pseudomonas aeruginosa* Isolated from Burns and Wounds Infections

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Abstract

During this study a total of (250) samples were collected from patients suffering from burns and wound infections from three hospitals in Baghdad during the period from (1/9/2019) to (30/12/2019). The isolates were firstly identified by a microscopic examination and their cultural characteristics on different selective media, then finely identified by VITEK-2 system. Results revealed that only 120 isolates (48%) of them were gave a typical morphological characteristic and biochemical test that belong to *P. aeruginosa* isolates. The molecular study included the detection of virulence genes *toxA* and exoS in *P. aeruginosa* isolates by colony PCR technique. Results revealed that *toxA* and exoS genes were found nearly in all *P. aeruginosa* isolated from burn and wounds infections, the *toxA* gene was detected in 27/30(90%) of isolates. Also, *exoS* gene was found in 26/30 (86.7%) of isolates.

Introduction

Burn wound infections (BWI) are one of the most common health problems and a major source of morbidity and mortality throughout the world. These infections lead to delay the healing of wound, increase the scarring and favor microorganisms proliferation, then result in invasive infections (WHO, 2006). A highly susceptible infection in burn wounds results from a loss of skin integrity and the reduction of immunity that mediated by the immune cells, also it's affected by the depth and extent of the burn injury, host factors, the virulence and amount of the microbial flora that colonizing the wound (Barajas-Nava *et al*., 2013). The skin is exposed to damage by thermal, mechanical and chemical factors, such as scalding or fires, flammable liquids and other sources of heat, electricity, sunlight, chemical , also may infrequently caused by nuclear radiation (Roshangar *et al*., 2019). The popular local signs of invasive burn infection are the appearance of focal, multifocal, or generalized dark brown, black or violaceous discoloration of the wound, including exchange of an area of partial thickness injury to full-thickness necrosis or necrosis of once viable tissue in an unexcused wound bed (Skariyachan *et al*., 2018). The

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most popular pathogens isolated from burn wounds are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter spp*., coliforms, *Streptococcus pyogenes*, also others pathogens like anaerobic bacteria (Bora and Dhar, 2018).

Pseudomonas aeruginosa is a member of the gamma Proteobacteria a class of bacteria. It's a gram-negative, aerobic, non-spore forming rod measuring about 0.5to 0.8 µm by 1.5 to 3.0 µm. It's belonging to the bacterial family Pseudomonadaceae (Zhapouni *et al*., 2009). The infections caused by *P. aeruginosa* are particularly problematic because it's resistant to antibiotics types and can acquire resistance to all effective antimicrobial drugs (Pachori *et al*., 2019). To successfully stay on host tissues, *P. aeruginosa* needs to attach, digest, infect the underlying cell layers and eventually spread to distant tissue sites to avoid nutrient exhaustion and to escape from the local immune surveillance (Laventie *et al*., 2019).

P. aeruginosa has many virulence factors such as LPS, alkaline Proteases, Elastase, Pyoverdine, Pyocyanin, Flagellum, Type IV Pili, Biofilm Formation, Exoenzym S and Exotoxin A. These are most important virulence factors that work together in different manners in induction of the immune system (Rocha *et al*., 2019). Exotoxin A (ETA) is an extremely toxic virulence factor released by *P. aeruginosa* into the extracellular medium by the T2SS system (Michalska and Wolf, 2015). *exoA* gene encodes (ETA), that causes ADP ribosylation of eukaryotic elongation factor 2 and leading to inhibition of protein synthesis, which has the same mechanism of action as the diphtheria toxin. ETA is a main virulence factor of *P. aeruginosa* in burn ward of hospitalized patients (Aljebory, 2018). *P. aeruginosa* exoenzyme S (ExoS) is a bifunctional cytotoxin 49-kDa, type III secretion (TTS) effector, which includes both a GTPase-activating protein (GAP) activity toward the Rho family of low molecular weight G proteins and an ADP-ribosyltransferase (ADPR) activity that targets LMWG proteins in the Rab, Ras, and Rho families. ExoS was encode on exoS gene (Rucks, 2005). Both Rho-GAP and ADPR activities will change host cell cytoskeletal function, that resulting in impaired cell migration and adhesion, disruption epithelial cell barriers and preventing wound healing (Sun *et al*., 2012).

Materials and Methods

Samples collection

A total of (250) samples were collected from patients suffering from burn and wounds infections from three hospitals in Baghdad: Al-Imamein kadhimein medical city, Al-Yarmouk teaching and

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Al-Karkh general hospitals during the period from (1/9/2019) to (30/12/2019). The samples were taken from both gender by a sterile cotton swaps, then directly diagnosed on different selective media and finally identified by VITEK-2 system.

Molecular study

In this study from a total of (120) isolates that related to *P. aeruginosa* only (30) isolates were screened for the presence of *exoS* and *toxA* virulence genes by using of a specific primers, as shown in Table (1-1). In colony PCR technique a single colony of bacterial isolate was taken from the nutrient agar plate and was added to the PCR mixture in place of purified template DNA (Aal Owaif, 2017). The PCR tube was consisted of primers, nuclease- free water and master mix. The total volume of PCR mixture was 20µl. Then the PCR tubes were transferred to the thermal cycler to initiate the amplification reaction according to specific program for each pair of primers, as shown in Table (1-2).

Table (1-1): Primers of virulence genes.

Bacteria	Gene	Primer sequence (5′→3′)	Product size (bp)	References
P. aeruginosa	***exoS***	F:CGTCGTGTTCAAGCAGATGGTGCTG R:CCGAACCGCTTCACCAGG	444 bp	(Fazeli & Momtaz, 2014)
	toxA	F:GGCTATGTGTTCGTCGGCTA R:TGATCGCCTGTTCCTTGTCG	487 bp	(AlKaaby, 2015)

Table (1-2): PCR amplification program of *toxA* and *exo*S virulence genes.

Steps	No. of cycles	Time (M:S)	Temperature
Initial denaturation	1 cycle	5 min	95 ºC

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Denaturation	30 cycle	30 second	95 ºC
Annealing		30 second	*toxA* =58 ºC
			exoS=55ºC
Extension		30 second	72 ºC
Final extension	1 cycle	7min	72 ºC

Results and discussion

Isolation of *Pseudomonas aeruginosa* isolates

Results revealed that among the total of 250 samples, only 120 (48%) isolates were given identical morphological characteristics that belong to *Pseudomonas aeruginosa* isolates. while the rest isolates were belong to other pathogenic bacteria from different genera such as *Staphylococcus aureus*, *K. pneumoniae*, *Proteus mirabilis*, *Escherichia coli*, *Acinetobacter baumannii* and *Enterococcus faecium*. 53 isolates (44.16%) of them were from male and 39 isolates (32.5%) were from female, their ages were ranging between (18-75) years for both gender. Also the results revealed that 28 isolates (23.3%) of them were from children between (1-10) years.

The results of isolation in this study has been matched with Honnegowda *et al*., (2019) who reported that a total of 737 wound swabs were taken from patients in the intensive care unit. Various M.o. has been isolated from these samples included *Pseudomonas aeruginosa* at ratio (35.3%), *Klebsiella pneumoniae* (28.5%), *Escherichia coli* (22.6%), followed by *Staphylococcus epidermidis* (20.8%) and *Proteus mirabilis* (9.3%). A study by Ghai *et al*., (2015) showed that *P. aeruginosa* was most frequent pathogen isolated from surgical wound infections, at ratio (55.45%) followed by *K. pneumoniae* (22.1%) also *A. baumannii*, *P. mirabilis* and *S. aureus* has been isolated at different ratio. A study by Aljanaby and Aljanaby , (2018) who was collected out of 295 burn swabs, 513 different bacterial strains were isolated from patients with burn infection stay at hospital. 335 isolates (65.3%) were gram negative bacteria and178 isolates (34.7%) were gram positive bacteria. *Pseudomonas aeruginosa* was one of the mainly common bacteria causing burn infection at ratio (41.34%), followed by methicillin resistant *S.aureus* (20.6%), *K.*

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pneumoniae (18.2%), *E.coli* (12%) and *A. baumannii* (7.2%).

A study by Chaudhary *et al*., (2019), who recorded that out of 109 swab were taken from burn wound patients. 55% of samples were taken from male while 45% of them were from female, their ages between (10-75) years. The most common bacteria isolated from these patients were *Pseudomonas aeruginosa* (24.95%), followed by *Staphylococcus aureus* (24.05%), *Klebsiella pneumoniae* (17.09%), *Acinetobacter* (15.19%), *Escherichia coli* (8.23%) and *Proteus* (4.43%). Also a study in Basra city in Iraq revealed that among a total of 267 burn samples collected, (44%) of them were from children under 10 years old because children at this age are likely to be more active and play in the kitchen so may exposed to fire places, stoves, hot kitchen appliances and hot liquid (Al-Shamsi and Othman, 2017). In general the reason for variation in bacterial isolated from burn wound infection in all studies may be due to the percentage of distribution of isolates that differs according to the place of clinical samples collection, environmental factors, virulence factors (Ogunseilan, 2005). Also the high prevalence rate of bacterial isolation may be due to factors related to the acquisition of nosocomial pathogens in patients with repeated or long-term hospitalization, prior administration of antimicrobial agents, complicating illnesses, or the immunosuppressive effects of burn trauma (Magneti *et al* ., 2013).

Detection of virulence genes by colony PCR

The results exhibited here show that 27/30 isolates (90%) have the *toxA* gene with amplified size 487 bp, as shown in figure (1-1). Also the results revealed that 26/30 isolates (86.7%) have the *exoS* gene with amplified size 444 bp, as shown in figure (1-2).

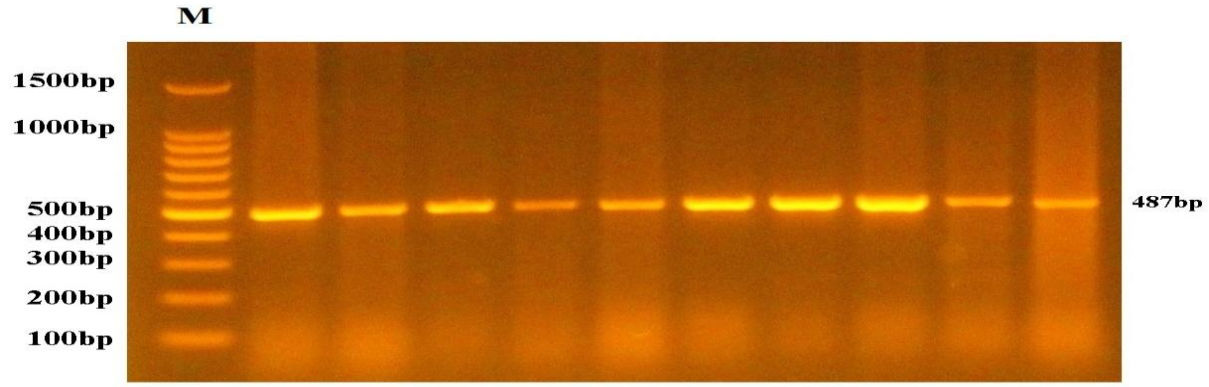

Figure (1-1):Colony PCR screening of *toxA* gene. (Agarose 1%, 10min, at 100 volts then lowered to 70 volts, 60min). Lane (M): 100bp DNA, while the other lanes represent *toxA*

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gene screened by *toxA*-F and *toxA*-R primers.

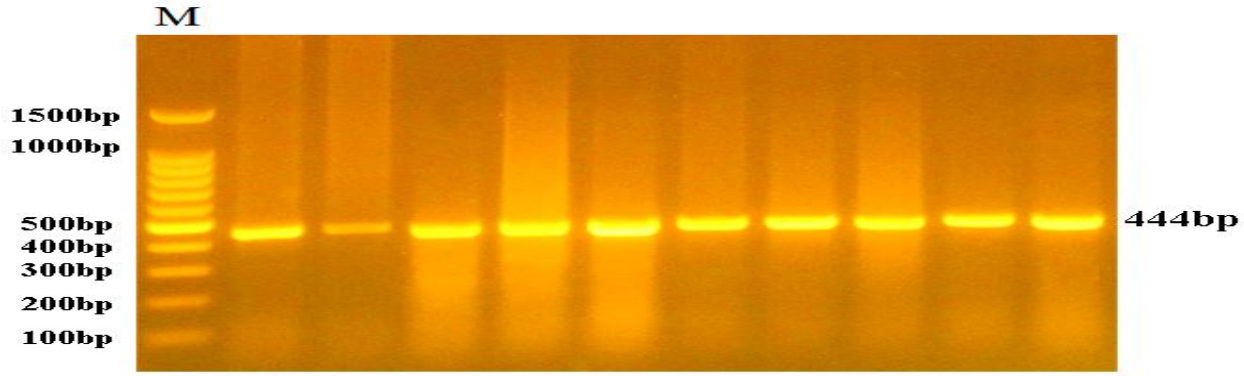

Figure (1-2):Colony PCR screening of *exoS* gene. (Agarose 1%, 10min, at 100 volts then lowered to 70 volts, 60min). Lane (M): 100bp DNA Ladder, while the other lanes represent *exoS* gene screened by *exoS*-F and *exoS*-R primers.

This result is consistent with the result of Chand *et al*. (2020) who recorded that (95.4%) of *P. aeruginosa* isolates carry the *toxA* gene. Exotoxin A encoded by the *toxA* gene that produced by *P. aeruginosa* inhibits protein biosynthesis by transferring an ADP-ribosyl moiety to elongation factor 2 of the host cell by stopping elongation of polypeptide chains. A study by Aljebory, (2018) from Iraq showed that *toxA* gene was found in (100%) of burns and wounds patients. Other studies by Haghi *et al*., (2018) and Aljebory, (2019) demonstrated that (97.8%) and (100%) respectively of the isolates were positive for *toxA* gene. Exotoxin A is an extracellular enzyme that is produced by most clinical strains of *P. aeruginosa* (Michalska and Wolf, 2015). The gene encoding Exotoxin A is found in 90-95% of *P.aeruginosa* (Matar *et al*., 2002). *toxA* plays an important role in the spreading of *P. aeruginosa* within the burned skin and had a special role in retardation of wound healing and contraction, and contribute to the overall virulence of *P.aeruginosa* in those burned patients (El-Din *et al*., 2008).

A study, in hospitals of Baghdad by Ali *et al*., (2020) who reported that *exoS* was the most frequent gene that was detected in (87%) isolates. Whereas study submitted by Al-Khafaji, (2014) from Iraq revealed showed that (80.0%) *P. aeruginosa* isolated from burn infection harbored *exoS* gene. The *exoS* gene is encoded for production of exoenzyme S (adenosine diphophate ribosyl tarnsferase) which is an important enzyme that transfer the ADP-ribose moiety from NAD+ to many eukaryotic cellular proteins and *exoS* is secreted by a type-III secretion system directly into the cytosol of epithelial cells for inhibiting the protein synthesis

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and disrupting endocytosis, the actin cytoskeleton, and cell proliferation, so resulting in apoptosis in the host cells (Fu *et al*., 1993; 2009;Sawa, 2014). *exoS* gene plays an essential role in burns infection by contributing dissemination of bacteria (Jabalameli *et al*., 2012), also the secretion of exoenzyme S by *P. aeruginosa* inhibits wound repair and effect on the host innate immune response to aid its colonization and its ability to cause injury (Stirling *et al*., 2006).

Conclusion: *P. aeruginosa* isolates were predominant bacteria isolated from patients suffering from burn and wounds infections. *toxA* and exoS genes were found nearly in all *P. aeruginosa* isolated from burn wounds infections.

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