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ORIGINAL ARTICLE

Molecular Detection of Virulence Genes in *Pseudomonas aeruginosa* Isolated From Patients with Burn Wound Infections from Burn and Plastic Surgery Department at Benha Teaching Hospital

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ABSTRACT

Background: *Pseudomonas aeruginosa have various virulence factors which participate in the bacterial invasion and toxicity. Different genes are responsible for their toxicity such as toxA, algD and plcN, exoS, lasB and plcH gene.* ***Objectives:*** *This work aimed to isolate and identify Pseudomonas aeruginosa that are prevalent in burn and Plastic Surgery Departments at Benha Teaching Hospital and to detect exoS, lasB and plcH virulence genes.* ***Methodology:*** *This work was done on 100 patients who had sustained burn injury from outpatients and inpatients of Benha Teaching Burn and Plastic Surgery Departments during the period from November 2016 to the end of April 2017. Burn wound samples were subjected to isolation and identification, exoS, lasB and plcH genes from 33 of Pseudomonas aeruginosa isolates were detected by using multiplex PCR.* ***Results:*** *this work showed that the most common cause of burn wound infections were Pseudomonas aeruginosa (44.7%). Among the 33 Pseudomonas aeruginosa isolates it was found that 26 (78.8%) of isolates have las B gene, 23 (69.7%) of isolates have plc H gene and 14 (42.4%) of isolates have exo S gene while the three genes were found in only 10 (30.3%) of isolates.* ***Conclusion:*** *: High percentage of isolated P. aeruginosa (78.8%) contains lasB genes This finding shows that P. aeruginosa was pathogenic , virulence and play an important role in burn infections*

INTRODUCTION

Burns are damage to the skin caused by variety of sources including chemicals, electricity, heat, sunlight or nuclear radiation. Thermal injury is a serious type of trauma which required care in a specialized units. It has been detected that approximately 2.5 million people sustain burns of which 100,000 are hospitalized and there are around 12,000 deaths per year due to thermal injuries[1]. The burn wound surface is a protein rich environment consisting of a vascular necrotic tissue that provides a favorable niche for microbial colonization and proliferation[2] .*P. aeruginosa* is found as an important colonizer of the burn wounds because it lives on moist wound surface and gains access to burn patients through cross contamination. It is considered as a major nosocomial infection threat to burn patients that shows of resistance against multiple antimicrobial drugs and frequently complicates the treatment of *P.aeruginosa* infection. This may lead to a high mortality rate in those patients.

P. aeruginosa also carries many intrinsic and acquired antimicrobial resistance traits that make infected bum wounds difficult to treat [3]. *P.aeruginosa* produces a number of cell-associated adhesions, alginate, pili, flagella, and lipopolysaccharide and extracellular elastase, exoenzyme S, exotoxm A, hemolysms, iron-binding proteins, leukocidins, and proteases virulence factors that mediate a number of processes, including adhesion, nutrient acquisition, immune system evasion, leukocyte killing, tissue destruction, and blood stream invasion [4]. It has been showed that pathogenicity of the bacterium depends on its virulence factors. Phospholipase C, exotoxin A, exoenzyme S and Pili are important for acute phase of disease. While, siderophoresand pseudocapsule of alginate are essential for chronic phase of *P. aeruginosa* infections[5]. Infections of *P. aeruginosa* are difficult to erase because of their high intrinsic resistance and their capacity to gain resistance to various antibiotics. las B is a zinc metalloprotease that has a high range of substrates, including elements of connective tissue such as elastin, collagen, fibronectin and laminin[4]. *P. aeruginosa* uses a type III secretion system to transport proteins such as ExoS directly into the cytosol of eukaryotic cells. ExoS, a bi-functional GTPase activating protein and ADP-ribosyltransferase, but has been connected to a higher incidence of dissemination to the bloodstream [6].

Pseudomonas aeruginosa hemolytic phospholipase C, PlcH, can degrade phosphatidylcholine and sphingomyelin in cell membranes. During infection,

PlcH can induce a proinflammatory response, inhibits oxidative blast in neutrophils, degrades pulmonary surfactant, and increases endothelial cell death[7].

METHODOLOGY

This work was done in Microbiology and Immunology Department, Faculty of Medicine, Benha university from November 2016 to the end of April 2017.

Subjects:

This study was conducted on 100 patients who had sustained burn injury from outpatient and inpatient of Benha teaching Burn and Plastic Surgery department. Their ages were ranged from 1 to 70 years. A written informed consent (in Arabic language) was obtained from the patients and patients parents (children patients) before Participation. Approval of the ethical committee for the work design was obtained from Faculty of Medicine, Benha University.

Samples:

Sample collection:

Burns were cleansed with sterile gauze and sterile normal saline. The sample was collected from either burn surface tissue or burn fluid (sampling by needle aspiration) with sterile cotton swabs.

Multiple samples from several areas were collected in order to get the most accurate assessment. They were taken before dressing changes and before administration of antibiotics whenever possible. The samples were transported immediately to the laboratory[8].

Isolation and identification:

Each sample was directly cultured on nutrient, blood and MacConkey medium agar plates without delay. The plates were incubated aerobically at 37ºC for up to48 hours[9]. Colonies were identified by biochemical reaction tests[14].

- Multiplex PCR:

DNA extraction:

Total DNAs of the bacterial isolates were extracted by the DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit,EU Lithuania, #K0721) according to manufacturer instructions. The extracted DNA was then stored at -20°C until further processing.

DNA amplification:

Amplification was done using Maxima Hot Start PCR Master Mix #K1051 (Thermo Scientific, EU Lithuania). The PCR mix contained 25ul of PCR master Mix, 2.5ul of each reverse primer and forward primer, 5ul of the template DNA and the amount completed with nuclease free water (5ul) to obtain a final volume of 50ul. *Biometra ,Germany* thermal cycler was used for amplification according to this program: initial denaturation at 95ºC for 4mins, 45 cycles of denaturation at 95 ºC for 30 s, annealing at 46 ºC for 30 s and extension at 72 ºC for 45 s, followed by final extension at 72ºC for 10 mins.

Sequence of primer (Biosearch technologies,USA):

las B
F:GGAATGAACGAAGCGTTCTCCGAC
R:TTGGCGTCGACGAACACCTCG
plc N
F:TCCGTTATCGCAACCAGCCCTACG
R:TCGCTGTCGAGCAGGTCGAAC 13
exo S
F:CGTCGTGTTCAAGCAGATGGTGCTG
R:CCGAACCGCTTCACCAGGC

DNA detection by agarose gel electrophoresis:

The detection of genes respectively by agarose gel electrophoresis was done according to [10].

10µl of each amplified DNA & 100bpladder (molecular weight marker) were separated on 2% agarose gel containing 0.3 mg/ml of ethidium bromide.

The bands were visualized by UV transilluminator (312 nm, Biometra, Germany), photographed & analyzed.

Statistical analysis:

Data were recorded and analyzed using STATA/SE version 11.2 for Windows (STATA corporation, College Station, Texas).

RESULTS

This work was conducted on 100 patients with burn wound infections from age 1 to 70 years. They were 60 females (60%) and 40 males (40%). 30 cases (30%) were Outpatients while 70 cases (70%) were admitted in Burn and Plastic Surgery Department in Benha Teaching Hospital.

Table 1 and fig 1 shows that out of 100 patients 60 cases (60%) were males and 40 (40%) were females. There was a significant differences between bacterial positive and negative patients regarding sex (p <0.001).

Table 1: Distribution of the studied group according to sex:

Sex	No. of samples (all patient)		No. of Isolates (positive patient)		% of Isolates From each group	Z test	P
	No	%	No	%			
Female	40	40	31	38.2	77.5	69.04	<0.001**
Male	60	60	50	61.8	83.3	90.93	<0.001**
Total	100	100	81	100	81.0	114.13	<0.001**

X^2= 0.531 P=0.466

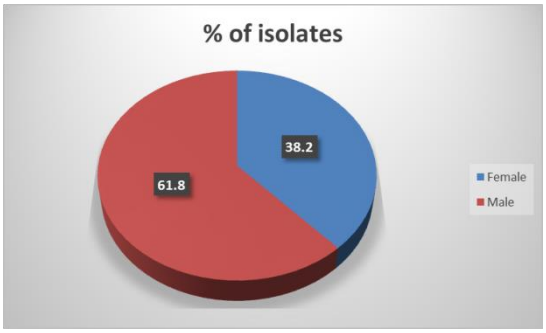

Fig 1: Distribution of the studied group according to sex

Table 1: There were significant differences in the ratio of bacterial positive and negative patients between Inpatients and Outpatients groups (P <0.001). There was also were a high significant difference in the proportion between Inpatients and Outpatients (P=0.017). The proportion of Inpatients was higher than Outpatients (70% *vs*. 30% respectively).

Table 2: Number and Percentage of cases collected from Outpatients and Inpatients at Benha University Hospital:

Persistent in hospital	No. of samples (all patient)		No. of Isolates (positive patient)		% of Isolates From each group	Z test	P
	No	%	No	%			
Outpatients	30	30	20	24.7	66.7	51.41	<0.001**
Inpatients	70	70	61	75.3	87.1	102.72	<0.001**
Total	100	100	81	100	81.0	114.13	<0.001**

X^2= 5.72 P=0.017*

Table 2 shows that the most common cause of burn infection was *Pseudomonas aeruginosa* 33 (40.74%) followed by *Staph aureus* 19 (23.46%).

Table 3: Types of the bacterial isolates

Isolated organism	Total no. of Isolates	% of isolates	Z test	P
Pseudomonas aeruginosa	33	40.74	51.35	<0.001**
Staph aureus	19	23.46	29.3	<0.001**
E.coli	13	16.05	19.84	<0.001**
Klebsiella pneumonia	11	13.58	16.69	<0.001**
Citrobacterfreundi	3	3.7	4.08	<0.001**
Proteus mirabilis	2	2.47	2.51	0.01*
Total	81	100		

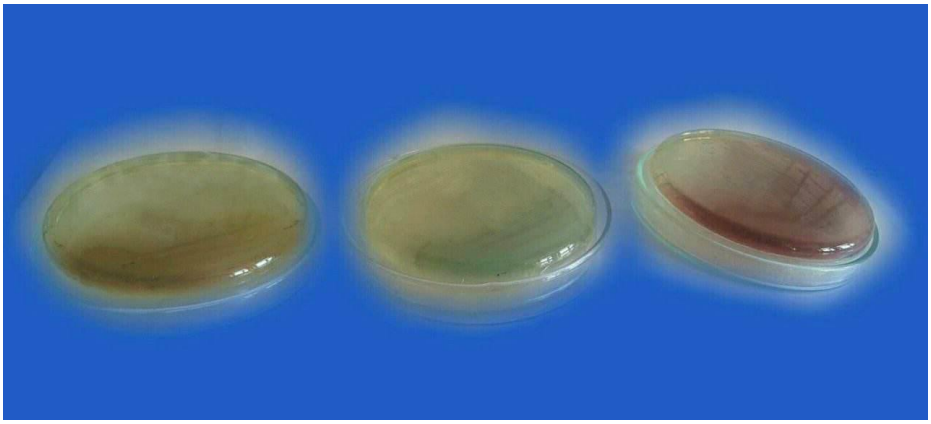

Fig 2: Different water- soluble pigments of *Pseudomonas aeruginosa* (pyoverdine (yellow – green) pyocyanin (blue-green), and pyorubin (red- brown) on nutrient agar.

Table 4: The Number and percentage of exo S, las B and plc H genes in *Pseudomonas aeruginosa* isolates by multiplex PCR technique:

Organism		Exo S		Las B		Plc H		Exo S , las B and Plc H	
	No.	No.	%	No.	%	No.	%	No.	%
Pseudomonas aeruginosa	33	14	42.4	26	78.8	23	69.7	10	30.3

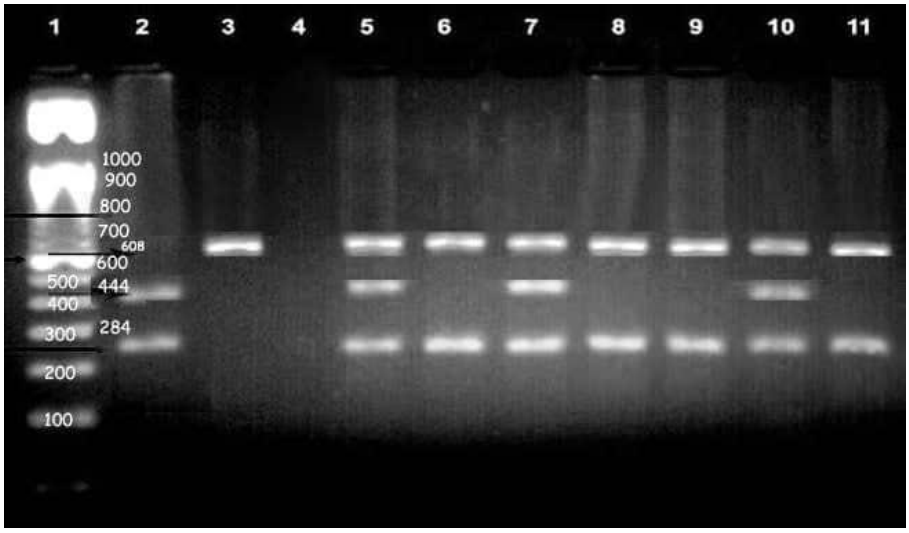

Figure 3: Gel electrophoresis of exoS ,las B and plc H genes

The product of primer of exo S gene was seen as band at 444 bp fragments, the product of primer of las B gene was seen as band at 284 bp fragments and the product of primer of plc H gene was seen as band at 608 bp fragments. (1) is a ladder. Lane (2) show a bands with molecular weight 444 and 284 bp (exo S and las B genes). Lane (3) show one band with molecular weight 608 bp (*plc H* gene). lanes (6,8,9,11) show a bands with molecular weight 284 And 608 bp (las B And plc H genes) .lanes (5,6,7,10) show a bands with molecular weight 444, 284 And 608 bp (exo S, las B And plc H genes) .Lanes (4) is negative .

DISCUSSION

Pseudomonas aeruginosa has various virulence factors which help the bacteria to colonize different parts in their host and the bacteria are an important cause of nosocomial and community-acquired infections worldwide [10] .

P. aeruginosa is one of the most important bacteria which can infect burn wounds in children [11]. Moreover, the bacteria have a number of virulence factors such as exotoxin A, elastase, alginate, phospholipase and exoenzyme S which are regulated by signaling systems[12].

Our study aimed to isolate and identify *Pseudomonas aeruginosa* that are prevalent in burns and to detect their virulence genes as exoS, lasB and plcH gene from Burn and Plastic Surgery Department at Benha Teaching Hospital

As regards sex, our study showed that males (60%) are more than females (40%). The high percent of male burn injured in our study could be due to increased exposure to activities. This finding agrees with Kamal et al.[13] who found that males (56.5%) were more affected to burn injury than females (43.5%). Also with Rafii et al.[14] who reported burns in 60% of males.

Other studies, E.G Forson et al.[15] reported that burn injuries were higher in females 32 (64%); compared to 18 males (36%) due to their daily activities in the kitchen, while most male subjects acquired burns at work.

In our study There was a statistically significant difference between Inpatients and Outpatients (P=0.017). The proportion of Inpatients was greater than Outpatients (70% vs. 30% respectively), this means that the staying in hospital enhance the microbes to colonize and cause infection in burn patients. This agrees with Kamal et al.[13] who found that the mean hospital stay was statistically significantly higher in patients with infection (14.20 days) rather than in patients with no infection (5.58 days) p<0.001

In our study we found that *Pseudomonas aeruginosa* was the most common isolated organism (44.74%) of cases, as it was considered as a major factor in the etiology of burn wound infection, followed by *Staph aureus* (23.46%) of cases is in agreement withMona et al.[8] and Hanaa and Rehal[18]who isolated *P. aeruginosa* from burn exudates in 66.7% of cases.

In our study we used the PCR technique to search the presence of exo S, las B and plc H genes in *p.aeruginosa* isolates and it was found that 26 (78.8%) of isolates had las B gene; 23 (69.7%) of isolates have plc H gene and 14 (42.4%) of isolates have exo S gene while the three genes were found in only 10 isolates (30.3%). These results match with Fatemeh et al.[16] .who reported that las B gene was found in (80%) of cases and plc H in (22%) of cases only.On the other hand Wolska and Szweda[17]reported that 162 strains of *P. aeruginosa*were isolated from infections of different parts of body and the prevalence of exoS (80%) was higher than the other virulence genes .

CONCLUSION

Our findings showed that High percentage of isolated P. aeruginosa (78.8%) contains lasB genes This finding shows that P. aeruginosa was pathogenic , virulence and play an important role in burn infections.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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