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Pseudomonas Aeruginosa Exotoxin A: Its Role in Burn Wound Infection, and Wound Healing

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic gram negative organism that can cause serious infections in burned patients. Exotoxin A (ETA) is the major and most lethal virulent factor produced by this microorganism. In this study, 97 burned patients were included. All patients had clinical local burn wound infection. Swabs and biopsies were taken from the wounds. Blood cultures were done for all patients. Pseudomonas aeruginosa producing ETA were identified by amplification of exotoxin A gene using PCR techniques. Study results suggested that exotoxin A (ETA) plays an important role in the spread of pseudomonas aeruginosa within the burned skin and the appearance of endogenous septicemia. Also, we proved that ETA had special role in retardation of wound healing and contraction. The conclusion was that ETA contributed to the overall virulence of pseudomonas aeruginosa in those burned patients.

INTRODUCTION

Infection of burn wounds is a serious problem leading frequently to death. Although presently more patients with burns die from pneumonia than from burn wound infection, burn wound sepsis remains an important infectious complications in this population **[1]**.

Thermal injury to the skin causes massive release of humoral factors, including cytokines, prostaglandins, vasoactive prostanoids, and leukotrines **[2]**. Accumulation of these factors at the site injury results in "spillover" into the systemic circulation, giving rise to immunosuppression. All arms of immune system are involved in immunosuppression **[3]**.

Burns predispose to infection due to damage of the protective skin barrier, facilitating entry of saprophytes, opportunists and pathogens **[4]**. In these conditions resulting from disruption of the skin mechanical integrity and generalized immunosuppresion micro-organisms can easily multiply and colonize wounds to high densities.

Pseudomonas aeruginosa particularly in economically developing countries is one of the most common and most important causes of serious infection in burn patients **[5,6]**. Pseudomonas infection contributes to high morbidity and mortality in burn patients. Despite advances in medical and surgical care the prognosis remains poor **[7,8]**.

Despite advances in surgical care and introduction of a wide variety of antimicrobial agents with antipseudomonal activities, life threatening infection caused by P. aeruginosa continue to be a common complication in burned patients and to contribute substantially to burn related morbidity and mortality worldwide. Exotoxin A (ETA) is the major virulent factor produced by most of the P. aeruginosa strains. It has been shown to play a major role in the pathogenesis of infection caused by this organism **[9,10]**. Exotoxin A (ETA) has proven to be toxic for mammalian cells **[11]**. Moreover it inhibits human granulocyte and macrophage progenitor cell proliferation **[12]**. Also, it was reported that ETA alter the production of tumour necrosis factor alpha (TNIFα) by human leukocytes **[13]**.

In this work, the prevalence organism in 97 patients with burn wound infection was studied. P. aeruginosa was considered the most common pathogen. We isolated the ETA which was expressed by ETA gene. Special diagnostic procedure was used that can rapidly and specifically detect P. aeruginosa ETA. We demonstrated the importance of detection of exotoxin A and its role in the pathogenesis of P. aeruginosa infection of the burn wounds. The necessity of ETA in the production of P. aeruginosa endogenous septicemia was investigated. Also relation of ETA production to burn wound contraction and healing was evaluated.

PATIENTS AND METHODS

This study was carried out during the period from January 2005 to December 2006. Ninty Seven patients were included in this study (71 females and 26 males). They were admitted to The Burn Unit, Mansoura Emergency Hospital. Mean age was 22.4 (15 to 40) years. The mean total burn surface area TBSA was 52.1% (range 30% to 80%), with mean full thickness burn of 31.4% BSA (range 20% to 50%). Patients with the following criteria were excluded: 1- Facial burns, 2- Suspected inhalation injury, 3- Chemical or electrical burns, and 4- Associated fractures or chronic illness.

Patients received immediate care and resuscitation according to Parkland's formula. Burn wounds were dressed with silver sulphadiazine. Local infection was suspected when there was black and dark discoloration in a previously clean appearing wound, early and rapid eschar separation, bleeding into subcutaneous tissue, and increasing edema into surrounding areas. The clinical signs suggestive of burn wound sepsis and septicemia were signs of disorientation, hyperpyrexia or hyperthermia, circulatory embarrassment. All patients had local signs of burn wound infection.

Swabs from burn wounds and samples from blood for culture were taken. Also wound biopsies were harvested from all patients. These procedures were done on post burn day 10. In burn wound biopsy small wedge of burned and subcutaneous tissue was excised. The bacteria were isolated from clinical samples (swabs, blood culture and wound biopsies) by standard microbiology procedures. Oxidative and oxidative fermentation tests for carbohydrate utilization were used for identification of P. aeruginosa.

In wound biopsy specimens, counting of colony forming units (CFU) per gram of tissue were performed after overnight incubation at 37ºC. Infection was diagnosed when number of CFU more than 10^5/gram of tissue.

Detection of exotoxin A gene:
(Done only for wound biopsy specimens)

DNA extraction: P. aeruginosa that was grown overnight in nutrient broth were centrifuged and the supernatant was transferred to another vial, then DNA was precipitated.

DNA amplification: Amplification reaction was performed by using a DNA thermal cycler. Diluted DNA samples were subjected to Polymerase chain reaction (PCR) amplification techniques. DNA samples were well mixed and slowly loaded into shots of submerged gel. Electrophoresis was done at current of 100 voltages and stained with ethedium bromide (0.5mg/mL). PCR products were visualized under UV light using the transilluminator FBTIV-88 (from fischer scientific, Pittsburg, USA) and photographed by digital camera (Olymbus, Cammedia C50C50, Japan) (Fig. 1).

Fourteen areas full thickness burn were chosen from those patients. Seven areas were infected with toxogenic (ETA) producing P. aeruginosa strains. The other 7 areas were infected with non toxogenic strains. Wound healing and contraction were assessed in these areas. Plannimetry based on colour changes in the edge of the wound was selected as the most accurate method for assessing surface area of the wound (Fig. 2). Measuring the wounds with a ruler were done every third day. Automatic calculation of the surface areas of the wounds were done and compared to each other.

Statistical methods:

Data were described as frequency, mean and standard deviation SD. Mann-Whitney-μ-test was used to compare burn surface area changes in both groups. There was high significant difference, *p* value=0.001 at three days and <0.001 later. These tests were run on an IBM compatible PC using SPSS statistical package for windows ver. 15 (SPSS Inc., Chicago).

RESULTS

In this study we observed that the 97 burned patients examined had local burn wound infection. Microbiological analysis of those patients with burned wounds showed that pseudomonas aeruginosa infection was the commonest pathogen (48.4%). Other various types of bacteria isolated from burn wound, swabs, biopsies and blood culture are mentioned in Tables (1,2,3). From this we notice that biopsies were more conclusive in detection of burn wound infection than swabs.

In P. aeruginosa isolates, detection of ETA gene by PCR and exotoxin A expressed from it, showed that toxogenic P. aeruginosa were more common than non toxogenic strains. Out of 47 P. aeruginosa isolates ETA gene was found in 42 specimens (89.4%). In the remaining 5 isolates, ETA gene could not be detected (Table 4).

There was correlation between exotoxin A (ETA), positive blood culture & presence of bacteraemia. Out of 37 cases (38.1%) of bacteraemia, 21 cases were P. aeruginosa producing ETA, while all remaining organisms caused bacteraemia with positive blood cultures in 16 cases (Tables 3,5).

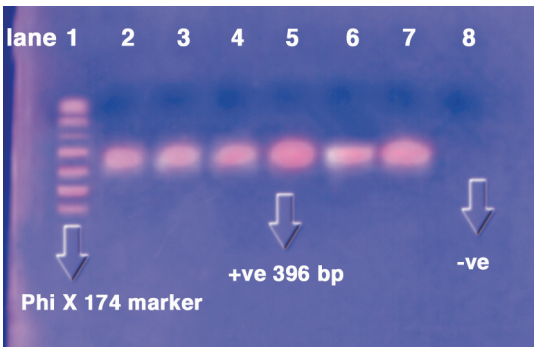

Fig. (1): Tox A gene detection by PCR in Pseudomonas aeruginosa isolates:
- Lane 1 → Molecular marker X 174.
- Lane 2,3,4,5,6 and 7 → +ve cases show 396bp bands.
- Lane 8 → –ve case.

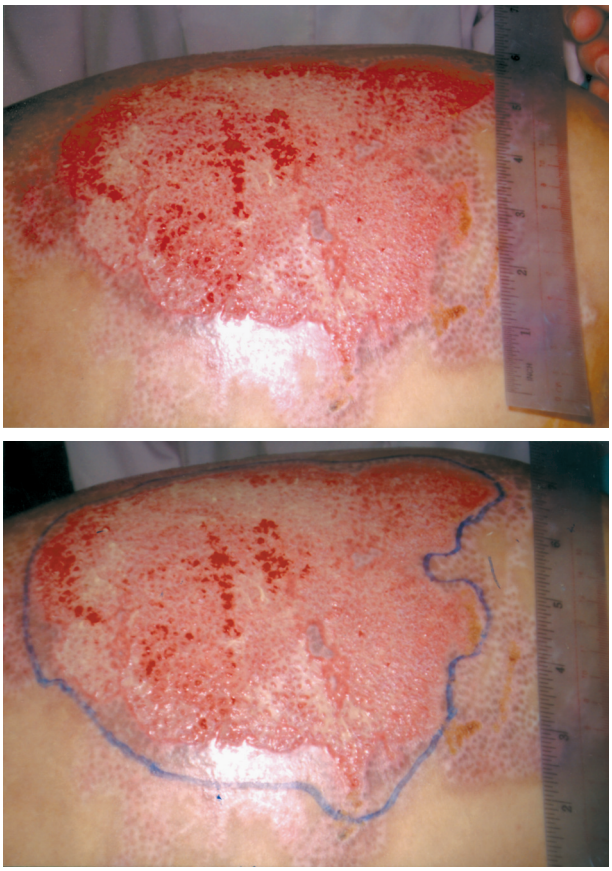

Fig. (2): Plannimetry: Measuring the wound with a ruler. Colouring the border of the defect to facilitate the colour changes. Automatic calculation of the surface.

In P. aeruginosa infection there was relation between bacterial count in wound biopsy, appearance of bacteraemia and positive blood cultures and production of ETA. We noticed that in 21 (50%) of patients infected with toxogenic P. aeruginosa, bacterial count was more than 10^5/gram of tissue and had positive blood culture. While non of patients infected with non toxogenic P. aeruginosa had bacterial count more than 10^5/gram with negative blood culture (Table 5). This gives a good idea about the virulent power of ETA that help the bacteria to invade deeper in the tissues and reaching blood stream.

As regard to wound healing and contraction, plannimetry was used for assessment. Contraction rates revealed obvious wound healing and closure in burn wounds infected with non producing exotoxin A P. aeruginosa, while wounds that colonize toxogenic P. aeruginosa strains showed retardation of wound healing and contraction (p<0.001) (Fig. 3).

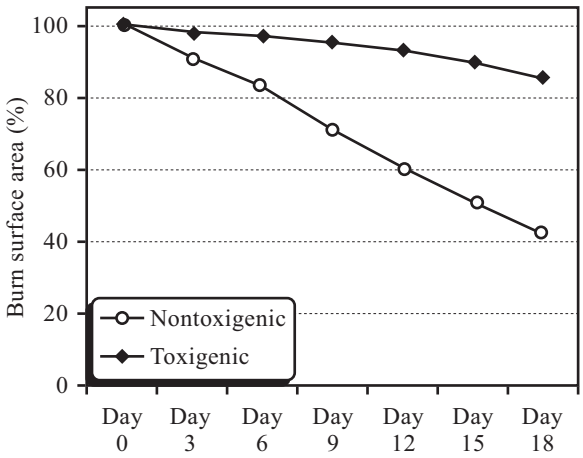

Fig. (3): Burn surface area changes in patients infected with nontoxigenic and toxigenic strains.

Table (1): Different bacterial isolates in studied groups.

Organism	No.	Percentage
P.aeruginosa	47	48.4
Klebsiella	9	9.3
E coli	13	13.3
Staph aureus	19	19.6
Coagulase-ve staph	2	2.1
Citrobacter	2	2.1
Proteus	3	3.1
Streptococcus	2	2.1
Total	97	100

Table (2): Bacteria isolated by both swab and biopsy.

Organism	Swab	Biopsy	Total
P.aeruginosa	44	47	47
Klebsiella	6	9	9
E coli	10	13	13
Staph aureus	14	19	19
Coagulase-ve staph	1	2	2
Citrobacter	1	2	2
Proteus	1	3	3
Streptococcus	1	2	2
Total	78 (80.4%)	97 100%	97 100%

Table (3): Bacterial isolates in blood culture of studied groups.

Organism	Positive blood culture (Septicaemia)	Total
P.aeruginosa	21	47
Klebsiella	2	9
E coli	0	13
Staph aureus	13	19
Coagulase-ve staph	0	2
Citrobacter	0	2
Proteus	1	3
Streptococcus	0	2
Total	37 (38.1%)	97 100%

Table (4): ETA gene detection by PCR in the P. aeruginosa isolates in studied groups.

Number of P. aeruginosa isolates	ETA gene detection by PCR	Percentage
47	42	89.4

Table (5): ETA in relation to biopsy bacterial count and development of septicemia.

	P. aeruginosa toxigenic strains	P. aeruginosa nontoxigenic strains	Total
Isolates with biopsy count less than 10/gram	21 (50%)	5 (100%)	26
Isolates with biopsy count more than 10/gram	21 (50%)	0 (0%)	21
Total number of isolates	42 (100%)	5 (100%)	47
Positive blood culture	21	0	21

DISCUSSION

Invasive burn wound infection is the main causative factor of sepsis, septic shock, and multiple organ dysfunction syndrome (MODS) in extensively burned patients **[14]** burn injury causes mechanical disruption to the skin. The usual skin barrier to microbes is replaced by a moist protein-rich, avascular eschar that foster microbial growth. The gram-positive nature of the microbial population does not alter immediately after burn but with passing of time 7-10 days post burn, more virulent gram-negative organisms replace the gram positive ones. The most virulent gram negative organisms is P. aeruginosa which is an opportunistic pathogen that causes serious infections.

In this study, 97 different bacterial isolates were cultured from 97 patients. Forty seven (48.4%) were P. aeruginosa isolates. P. aeruginosa was found to be the commonest pathogen causing burn wound infection in Burn Unit of Mansoura Emergency Hospital. This agreed with other studies which reported that the most prevalent bacteria in many burn centers were P. aeruginosa organisms **[5,6,7]**.

Pseudomonas aeruginosa is an opportunistic pathogen that frequently causes severe infection and septicemia in immunocompromized hosts especially in patients with major burn injury. P. aeruginosa septicemia carries a higher fatality rate than that caused by any other gram negative bacteria **[18]**. Lipopolysaccharide (LPS) of this pathogen should be an important virulent factor **[19]**. However, LPS isolated from the cell walls of P. aeruginosa is not as toxic as those isolated from enteric bacilli **[19]**. Therefore, it is clear that additional factors are involved in the virulence of this organism. P. aeruginosa produces a large number of extra cellular products which are much more toxic than LPS and have been shown to play a role in the pathogenesis of the infections caused by this organism **[9,19]**.

Exotoxin A (ETA) is one of the major virulent factors produced by this organism. P. aeruginosa ETA was first discovered and purified by Liu and associates **[20]**. Since then, ETA had been proven to be toxic for a wide variety of mammalian cells in vitro **[21]**, and lethal for many animal species **[22]**. In mice, ETA is approximately 10,000 times more lethal than lipopolysaccharicle from P. aeruginosa **[23]**.

ETA is a protein toxin that inhibit polypeptide synthesis through ADP ribosylation of elongation factor 2, as does diphtheria toxin leading to cell death **[24,25]**. Its cytotoxic activity extends to a wide variety of mammalian cells. ETA has been shown to inhibit proliferation of human granulocytes and macrophages **[12,21]**, to alter the production of tumour necrosis factor alpha (TNFα) by human leukocytes, and inhibit interleukin-1 production by murine peritoneal macrophages **[26]**. It induces murine cytotoxic T lymphocytes **[27]**. These data indicate that ETA may play a role in the pathophysiology of P. aeruginosa septicemia, and death among burned patients **[18,28]**.

In this research out of the 47 P. aeruginosa isolates, ETA gene was found in 42 (89.4%) isolates by PCR tests. Similarly, vasil and co-workers reported ETA production in approximately 90% of

111 isolates of P. aeruginosa by assaying for ADP ribosyl transferase activity **[29]**. Khan and Cerniglia reported that ETA gene was found in 93% of P. aeruginosa by PCR **[30]**.

The presence of ETA producing pathogens has special relation to appearance of bacteraemia and positive blood culture. Our results showed that out of 42 patients infected with P. aeruginosa toxogenic strains, 21 had bacterial count in wound biopsy more than 10^5 CFU/gram of tissue, positive blood culture, and septicemic manifestations. While non of patient infected with P. aeruginosa not producing ETA had bacterial count more than 10^5 CFU/gram.

In agreement of our results Woods and co-workers, reported that clinical blood isolates of P. aeruginosa in burned patients produce a large amount of ETA in vitro compared with isolates obtained from other infection sites **[31]**. Cross and associates reported that patients with P. aeruginosa septicemia had a better prognosis when their antibody titres to ETA were high **[32]**. Hirakata and associates in their experimental study showed that the clinical bacteremic isolates of P. aeruginosa caused higher mortality rate in mice than did strains which does not produce ETA. They also proved that ETA affects one or more steps in the bacterial colonization of the intestine, with subsequent invasion of the bloodstream and resistance against phagocytosis by Kupffer cells **[23]**. Also the same authors in previous study reported that toxogenic P. aeruginosa resists blood clearance in mice in vivo and kupffer cell association in vitro and that systemic bacteraemia consequently occurs **[33]**.

Another study suggested that ETA played an important role in the horizontal spread of P. aeruginosa within the burned skin and in the dissemination of P. aeruginosa within the bodies of burned-and-infected mice and contributed to overall virulence of P. aeruginosa in an animal model **[34]**. Interestingly, Hirkata et al., reported that erythromycin which has no direct antimicrobial activity against P. aeruginosa, enhanced the survival rate of mice with septicemia. This can be explained by the fact that suppression of ETA by erythromycin seemed to be one of the mechanisms in the protection of mice against P. aeruginosa septicemia **[35]**.

All of the above data and our results suggested that ETA may play an important role in the occurrence of P. aeruginosa bacteremia and its lethality in burned patients. ETA contributes to dissemination of P. aeruginosa within the body of burned patients and its horizontal spread within the burned skin. It is also responsible for overall virulence of P. aeruginosa. This study results could put us on the line to control severe burn wound infection caused by P. aeruginosa organisms and any sequalae or complications arising from it.

In addition to these results, we reported the application of a PCR procedures that can be used rapidly and specifically to detect P. aeruginosa strains that produce ETA in burns wounds by amplifying ETA structural gene.

Wound healing is a major concern in treatment of burn injuries. Our study showed direct relation between inoculation of the burned wound with ETA and delay in healing process as measured by the rate of wound contraction using plannimetry. Toxogenic P. aeruginosa isolates were associated with retardation of wound healing, while non toxogenic P. aeruginosa isolates were associated with more rapid rate of wound healing and contraction $p<0.001$.

Several studies have been conducted in order to study the effect of P. aeruginosa ETA on wound healing and contraction, and supported our results. Heggers and colleagues in their experimental study investigated the role of exotoxin A in the retardation burn wound healing and contraction. They reported that ETA retards wounds contraction. Contraction rates revealed significantly retarded closure in the animals treated with ETA compared with the rats of the non infected control groups ($p<0.05$). Animals treated with ETA plus anti ETA showed contraction rates identical to control groups. These data suggest that ETA retards wound healing and that neutralization of the toxin restores the normal healing process **[36]**. Azghani reported that epithelial wound healing was incomplete, and wound closure and epithelial junctional integrity were never achieved in the presence of P. aeruginosa ETA **[37]**. Christopher and associates reported that ETA inhibits corneal wound healing and promoting the maintenance of infection on corneal epithelium. This explained by the fact that ETA inhibit the production of growth factors which are linked to the regulation of epithelial migration, growth and adhesion **[38]**.

In conclusion, this study proved that P. aeruginosa that express ETA were the most common and toxic organism in burn units causing infection to burned wounds and leading to septicemic manifestations. Early diagnosis and detection help to combat its toxicity. Also presence of ETA producing organisms had special relation to retardation of wound healing and contraction.

This work and other similar studies initiate us to do further studies in order to understand the immunochemistry of ETA and to identify the immunodominant neutralizing epitopes of this molecule. Such studies are essential for the development of immunotherapeutic approaches for treating burn wound infections caused by toxin producing strains of P. aeruginosa and for elucidating the structure function relationship of ETA. It is also of great value for investigators interested in developing ETA derived immunotoxins. We believe that a combination of antibiotic antitoxin antibodies therapy could be beneficial to immunosuppressed patients (e.g. burned patients) in combating P. aeruginosa infection.

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