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Experimental Study of Pathological and Some Immunological Aspect of Infection *Pseudomonas aeruginosa* bacteria and Exotoxin in Rabbits

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Article History	Abstract
           	Pseudomonas aeruginosa is an opportunistic pathogen which infect immunocompromised patients. bacteria produce large types of virulence factors that serves its pathogenicity. The exotoxin A is major toxic extracellular virulent factor produced by P. aeruginosa. To clear the effect of exotoxin A and P. aeruginosa , Bacteria suspension and Exotoxin A extraction were injected intraperitonially in four group of rabbits, the result show there was significant decrease in total leukocyte count in all groups specially after 7 days from injection of Bacteria suspension and Exotoxin A also there is increase in neutrophilia percentage is the same period, the bacteria suspension and toxin A are capable alone or in both to activated phagocytosis, and produce neutralizing antibodies and produce pathological and immunological effect in liver spleen , kidney and lung and this suggest that toxin A and P. aeruginosa bacteria can effect in some immunological and pathological aspect when injected in experimental rabbit. **Keywords:** *Immunological, Bacteria,*

1. Introduction

Pseudomonas aeruginosa is a classical opportunistic pathogen which infect immunologically compromised patients. These pathogens are aerobic Gram-negative bacilli, motile, oxidase and catalase positive (**1,2,3**). They occur widely in nature including water, vegetation, soil and frequently associated with animals. The organism is resistant to wide variety of antibiotics and because its relatively resistance to drugs, it may persist in infectious processes while other more susceptible organisms have been eliminated by treatment (**1,3**)

Pseudomonas aeruginosa usually requires a substantial break in first –line defenses to initiate infection which can result from bypass of cutaneous or mucosal body barriers, disruption in the protective normal mucosal flora due to uses of broad-spectrum antibiotic, or alteration of immunological defense mechanisms (**2,3**).

Pseudomonas aeruginosa produce a large types of virulence factors that serves its pathogenicity. Since bacterial adhesion is the first stage in the infection process, the pili play major role in the *Pseudomonas aeruginosa* pathogenesis. In addition to the pili, alginate has been implicated as an adhesion factor of Pseudomonas *aeruginosa*, also Lipopolysaccharide is another factor plays a critical role in the *Pseudomonas aeruginosa* pathogenesis (**1**,4). These microorganisms also produce several extracellular enzymes and toxins that after colonization can cause extensive tissue damage, blood stream invasion, and then dissemination. These factors include exotoxin A, siderophores, exoenzymes S, alkaline protease, and phospholipase C. (**1,2,4,5**)

The major toxic extracellular virulent factor produced by *Pseudomonas aeruginosa* is exotoxin A, the exotoxin A classified as one number of the family of enzyme called the mono-ADP-ribosyl transferases (ADPRTases). toxicity of this single –chain 660 kDa poly peptide is due to ability to inhibit protein synthesis in susceptible cell by catalyzes ADP-ribosylation and inactivation of elongation factor 2 resulting to inhibition of protein synthesis and cell death (4,6,7**)**

Phagocytosis of bacteria by polymorphonuclear cells (PMNs) is enhanced by opsonin antibodies to bacterial envelop structure. these antibodies represented one important class of opsonins, complement

is opsonic in Gram negative bacteria include *Pseudomonas aeruginosa* but complement seems more effective in the opsonization of rough strain than the smooth clinical isolate (**8**). the experiments with *Pseudomonas aeruginosa* improve the evidence of a role for PMNs in efficient protection with cell-surface – derived antibodies (**8,9,10,11**). the aim of this study was to investigation and compare of some immunological and pathological effects of injection of *Pseudomonas aeruginosa,* bacterial exotoxin A, and a combination between them in rabbits.

2. Materials And Methods

Bacterial strain: *P. aeruginosa* that used in this study was isolated from human wounds. Isolation and diagnosis were done using morphological, cultural, and biochemical tests and the identification was confirmed by the API 20 E system.

Animal model: rabbits 3-4 months old were used in this study. Animals were housed in metallic cages and each rabbit was tested bacteriologically by making faces culture prior to the study and rabbits that were not carrying *P. aeruginosa* were used.

Bacterial suspension: *P. aeruginosa* was inoculated in Trypticase soy broth (Oxoid) with and incubated at 37°C for 24 hours. Different dilutions of the broth were made using PBS to give (6.5×10^5 CFU/ml) using standard method as described by (**12**).

Exotoxin A extraction: This procedure was described by (**13**) for the extraction of exotoxin A from *P. aeruginosa* isolates, stock cultures were streaked on Trypticase Soya Agar (TSA) and incubated overnight at 30°C. The isolated colonies were picked, streaked on Trypticase Soya Agar (TSA) slants, and incubated overnight, the growth was suspended in Trypticase Soya Broth which contained 15% (v/v) glycerol and was stored in aliquots at -70°C. For each experiment an aliquot was thawed and used to inoculate a Trypticase Soya Agar (TSA) slant, after an overnight incubation at 30 °C, growth from this slant was used to inoculate 40 ml of Trypticase Soya Broth (TSB) with nitrilotriacetic acid (NTA) that was added to this in final concentration of 5 mM, this concentration of nitrilotriacetic acid (NTA) has no effect on the growth of several *P. aeruginosa* strains tested, it is also inhibits protease production and enhance rather than depressed exotoxin production by protease- producing strain. Cultures were shaken at 250 rpm for 24 h. at 37°C in a shaking incubator and centrifuged for 20 minutes at 10.000 rpm, and the supernatants were filter-sterilized with 0.45 µm membrane filters.

Experimental design: Eight rabbits were used in this study, the animals were divided into four groups (tow animals for each group),animals of the first group (T1) received by 1ml of exotoxin A by intraperitoneal injection and animals in second group (T2) received 1 ml from bacterial suspension (6.5×10^5 CFU/ml) (**14**) intraperitoneally, while animals in third group (T3) received 1ml from both *P. aeruginosa* bacterial suspension and exotoxin A by the same way, the fourth group (T4) is injected with normal saline by the same way and consider as control Blood was collected from all groups in 7, 14, 21 days after injection for serological and determined number of white blood cell, also organs (liver, spleen, kidney and lung) were taken in day 21 for Histopathological study.

Determination of Total leukocyte count: hemocytometer slide was used to determine total leukocyte count; we used this equilibrium to find total white blood cell. (15)

Total white blood cell /ul = mean of total white blood cell in 4 esquire mm x 200

Determination of leukocyte percentage: A thin film from blood was prepared, fixed by alcohol stained with Gimza then 100 white blood cells were counted and percentage calculated (**16**).

Determination of macrophage activity: This procedure was described by (**17,18**) ,20 µl from blood mixed with same volume of nitrobluetetrazolum bromide (MTT) and incubated in 37°C for 30 minutes then thin blood film was made and stained with Wright stain and examined under oil immersion lens. The macrophage contain purple formazan granules was checked and the percentage of phagocyte index was calculated by:

$$\text{phagocyte index} = \frac{\text{number of macrophages contain formazan granules.}}{\text{total number of macrophages in slide}} \times 100$$

Agglutination power of animal's serum: a drop of serum from rabbit was mixed with drop of bacterial suspension (6.5×10^5 CFU/ml) in slide and determined the agglutination if occurred.

Histopathology: Tissue samples from Rabbits liver, spleen, kidney, and lung were collected, and the samples were fixed in 10 % neutral buffered formalin and processed for paraffin

embedding. The histopathological sections (3-5 µm) were stained with hematoxylin - eosin. The slides were coded and examined (19).

Static analysis: The statistical analysis of the results was carried out with the SPSS computer programme. the significant between Specific group differences were checked by Duncan's multiple range test. The values are expressed as mean and standard deviation using significant level of $P < 0.05$. (20).

3. Results and Discussion

Clinical sings: animals in all groups were showed singes of dullness and loose of appetite, decrease in food intake.

Determination of total leukocyte count & **leukocyte percentage**: Rabbits total leukocyte count showed significant decrease after 7 days of injected in all group toxin T1, bacteria T2, bacterial and toxin T3 in compare with control T4 followed by increase in number in all group after 14days and high significantly appear in T2, and T3. after 21 days all groups show decrease in total leukocyte number but without showing significant difference between them. the effect of time inside each groups showed significant difference between 7 days in all groups with 14and 21 days in same group (Digram1).
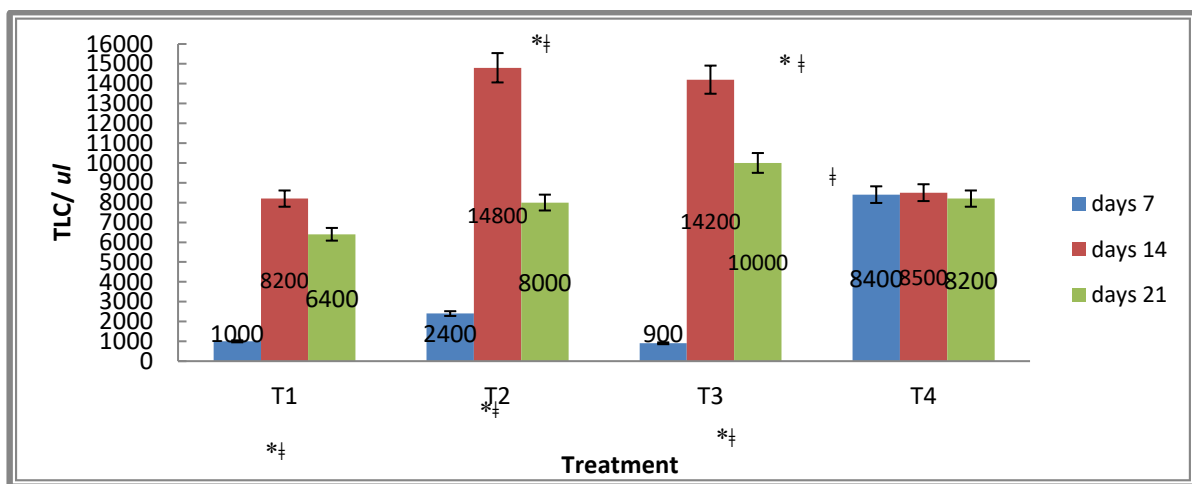
Data number represented TLC/*ul* means ± stander erorr . * Represented significant diffrent between groups; ǂ represented represented significant diffrent between time inside the same group. $P < 0.05$

**Daigram1:** effect of *P. Aeruginosa bacterial, toxin A* and both *injection* in total l white leukocyte count

The rabbit neutrophilia percentage increase significantly in 7 days in group T1 and T3 to reach (58, 57) respectively while other group showed no significant difference in 7, 14,21 days also there is significant effect of number of neutrophilia in day 7 and 21 in group T1 and T3 respectively when compeering between time inside same group (Diagram 2).
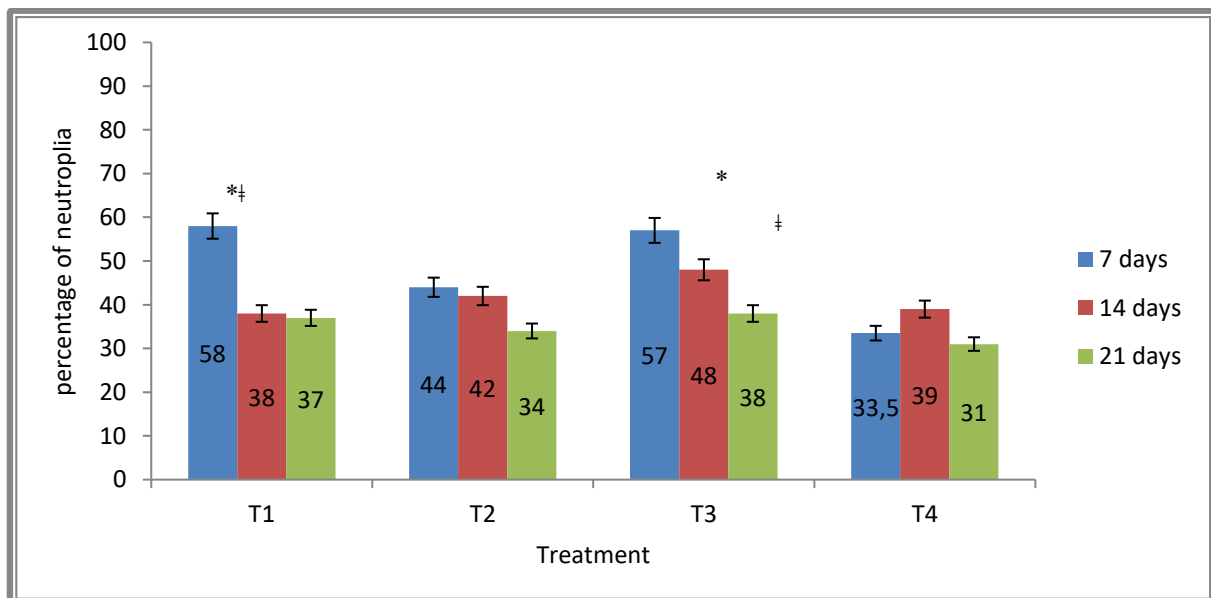
Data number represented percentageof neutroplia cells means ± stander error. * Represented significant different between groups; ǂ represented significant different between time inside the same group. $P < 0.05$

Diagram 2: effect of *P. Aeruginosa bacterial, toxin A* and both *injection* in percentage of neutrophia cell

lymphocyte percentage showed significant decrease in number specially after 7 days in T1 group from other T2, T3, T4 groups there is no significant difference between time in same group except in day 7 in t! Group which showed significant deference from 7,21 days in same group (Daigram3)
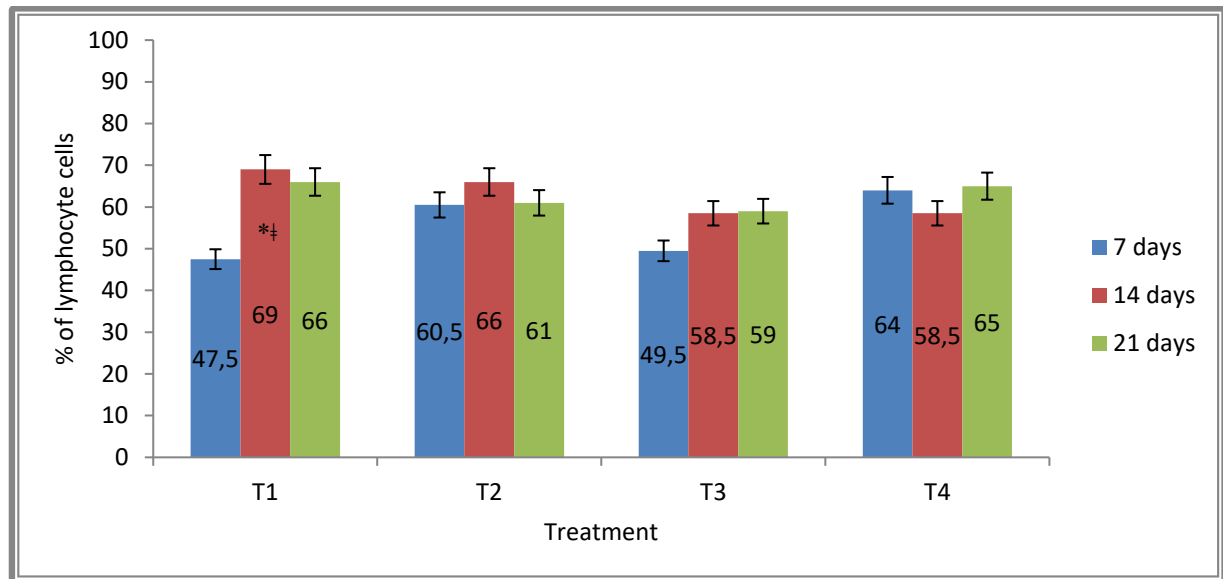
Data number represented percentage of lymphocyte cells means ± stander error. * Represented significant differences between groups; ‡ represented significant differences between time inside the same group. P < 0.05

Diagram 3: effect of P. Aeruginosa bacterial, toxin A and both injection in percentage of lymphocyte cell

Determination of macrophage activity: there was an increase in macrophage activity with the time in all groups, but toxin group show high percentage (Table 1) specially after 21 days of experimental.

Table 1: Determination of macrophage activity by Nitro bluetetraziolum bromide

group	Period days	Phagocyte% Contain formazan granules	Phagocyte% Not Contain formazan granules
T1	**7**	**73.2**	**26.8**
	14	**82.5**	**17.5**
	21	**85**	**15**
T2	**7**	**66.3**	**33.7**
	14	**81**	**19**
	21	**70.3**	**29.7**
T3	**7**	**70.5**	**29.5**
	14	**84.2**	**15.8**
	21	**71.4**	**28.6**

Power of agglutination of animal's serum: there was increased in power of serum agglutination with time in all group but bacterial and toxin group showed high level of agglutination reaction. (Table 2)

**Table 2:** Power of agglutination of animal's serum

Day \ Group	7	14	21
T1	++	++	+++
T2	+	+	++
T3	++++	++++	+++++

Histopathology:

Organs of animals show several changes which appear in (Table 3, Diagram 4).

Table 3: Histopathological changes occur in experimental animals' organs

Organs / Group	liver	Spleen	Kidney	Lung
T1	infiltration of inflammatory cell in portal area, congestion of blood vessels, cell swelling, fatty change	Depletion of lymphoid tissue	Heamorrhage, cell swelling, vacuolar degeneration	Pneumonia, infiltration of inflammatory cell in alveoli
T2	Vacuolar degeneration, congestion of blood vessels	Depletion of lymphoid tissue	Cloudy swelling	No changes
T3	infiltration of lymphocyte cell, congestion of blood vessels	Depletion of lymphoid tissue	Cloudy swelling, Heamorrhage	Pneumonia, infiltration of inflammatory cell in alveoli
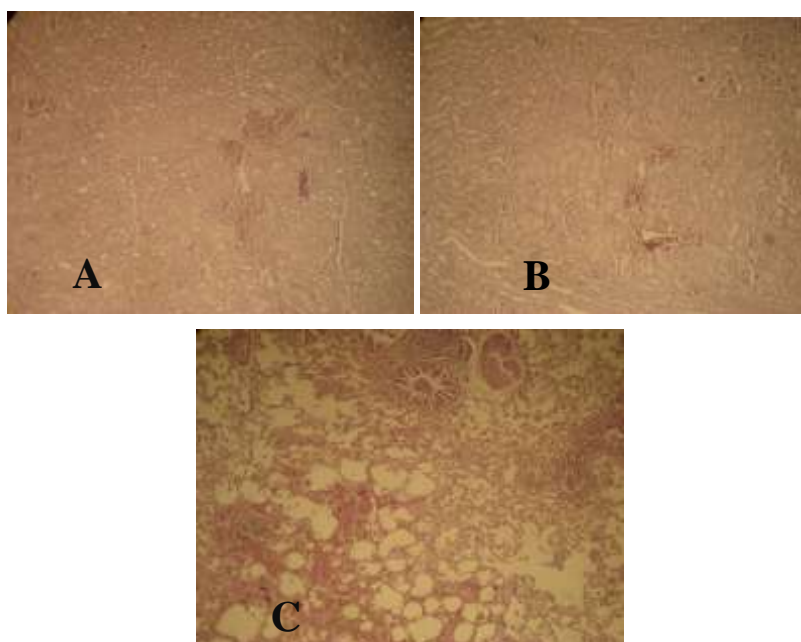
Diagram 4: Histopathology of animals Organs (A: Kidney tissue suffer from Cloudy swelling and Hemorrhage in bacteria &toxin group 90x; B: Kidney tissue suffer from cell swelling and Hemorrhage with vacuolar degeneration in toxin group 90x; C: lung tissue suffers from Pneumonia with infiltration of inflammatory cell in alveoli in toxin group 90x)

Antimicrobial host defense in animal is regulated by complex interplay between cellular or humoral effectors mechanisms or combination between them and that depend on bacterial type and its virulence (**21,22**). It's essential to increase TLC for clearance of virulent bacteria when enters the body. Because there is evidence that leukopenia animals may suffer from *P. aeruginosa* infection (**10**).

In the present study diagram 1 show that total leukocyte cells while total white blood cells was decreased significantly at day 7 after injection of toxin ,bacteria , bacteria and toxin to reach 1000, 2400 and 900 respectively this could be due to early response to antigens which caused cellular migration to the site of injection then cells was stimulated the cellular mechanisms of the body to produce antibodies and also the effect of toxin produced by *P. aeruginosa* especially toxin A which has been reported as having immunosuppression effect (**3**). also there are decrease in total white blood cells in the day 21 post inoculation in each group in correlation with time after reaching the peak in 14 day and in significant level in T2 and T3 post infection and this this may be due to immune response to this antigens specially

exotoxin A and stimulation of immune cells system which is essential for clearance of bacteria from the body and formation of antibodies against *P. aeruginosa* antigens (**23**), in diagram 2 study of neutrophil percentage T1 And T3 show high significant increase number of neutrophils and this may result from action of neutrophilia to neutralized and kill *P. aeruginosa* bacteria and toxin, neutrophilia were recruited in site of injection early and apoptosis bacteria then killing it by direct oxidative and non-oxidative mechanizes (**24,25,26**)

Leukocyte show decrees in day 7 in toxin group, and this may be due to cooperation between antigen presenting cells and lymphocytes to produce specific antibody binding antigen (22). From table 1 The percentage of Phagocytosis was increased at the day 14 after administration of toxin, bacteria, bacteria and toxin and reached to 82.5%, 81% and 84,2% respectively. this indicated that cell become active and have more power for phagocytosis after binding of microbial product to cell –surface receptor (**24**), also we seen that toxin could be enough to stimulate macrophage to do its activity rather than bacteria alone or combination between bacteria and toxin. Because the exotoxin induces apoptosis of phagocytosis cells include macrophage, neutrophils, dendritic cells (**25**).

In table 2, the power of agglutination was increased with time to reach high level in day 21 in all groups, but animals received bacterial and toxin injection were showed more agglutination power than those received bacterial and toxin alone and this may be because more epitopes (specific and common) are likely present and could induce protective immunity against *Pseudomonas aeruginosa* infection (27)**.**

Histopathological examination table 3 was showed that there is delectation of lymphocyte from spleen, and this is due to rapid attraction of immune cell to site of injection. also, the liver, kidney and lung showed microscopic changes which is due to effect of toxin injection and bacterial infection with the largest effect for toxin group. (**3**) refereed that the pureed exotoxin A injection is highly lethal for mice and animals, and it is acting as major systemic virulence factor of *P. aeruginosa* that were causes tissue damage and necrotizing effector site of bacterial invasion also its inhibition of protein synthesis inside cells which lead to death (**24**).

4. Conclusion
We concluded that *P. aeruginosa* bacteria and *P. aeruginosa* exotoxin A, cause changes in total leukocytic count and also have some effect on neutrophil and lymphocyte number, *P. aeruginosa* exotoxin A also immunogenic and produce antibodies but less power that complete bacteria do, and exotoxin A produce pathological and immunological effected on liver, spleen, kidney, lung.

Acknowledgements

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