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Comparison study between a crude bacterial suspension and purified pseudomonal alkaline protease enzyme effect on the rabbit interleukin – 2 following bacterial keratitis

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Abstract
The present study was design to determine and evaluate the inhibitory effect of crude bacterial suspension and pseudomonas alkaline protease enzyme on the rabbit interleukin 2. Eight serum samples were collected from male rabbits; four rabbits were infected with a crude bacterial suspension of *Pseudomonas aeruginosa* at a dosage of 500µl. The remaining four rabbits were infected with pure pseudomonal alkaline protease enzyme by the same dosage bacterial suspension. Serum samples from two infected rabbits groups were examined by ELISA and used for the detection of the level of IL2. The results showed that the serum IL2 level of bacterial suspension treatment group significantly reduced by a mean of 14 ug/dl after treatment. The treatment effect in this group was evaluated as a strong effect. Whereas the serum IL2 level among the enzyme treatment group showed no statistically significant difference from a mean change of zero after treatment.

Keywords: Pseudomonal alkaline protease, interleukin 2 and alkaline protease, ELISA, alkaline protease

1. Introduction
Pseudomonas aeruginosa, is an extracellular opportunistic pathogen, utilizes two major mechanisms to evade the host defense system [1]. One of these mechanisms is the production of a large number of extracellular products, such as proteases, toxins, and lipases [2]. The two proteases, alkaline protease and elastase, inhibit the function of the cells of the immune system (phagocytes, NK cells, T cells), inactivate several cytokines (IL-1, IL-2, IFN-r, TNF), cleave immunoglobulin's and inactivate complement [reference needed]. Inhibition of the local immune response by bacterial proteases provides an environment for the colonization and establishment of chronic infection [3]. Alkaline protease (APR) (peptidyl – peptide hydrolyses) is a 36 -38 kDa and the major virulence factors produced by *Pseudomonas aeruginosa* (*P. aeruginosa*) [4]. It was known to mediate bacterial binding to corneal surface and degrades fibrin [5]. It interfere with host defense systems by degrading complement component (C1q, C3), IgG, IFNy, IL- 1, IL-2 and TNF in conjunction with elastin [5]. At present APR are produced by a wide range of microorganisms, including bacteria, molds and yeasts, its large proportion being derived from bacillus strain [6, 7, 8]. Studies showed that *P. aeruginosa* alkaline protease interferes with classical and lectin pathway-mediated complement activation via cleavage of complement components C2 [9].
Structural comparison has revealed that APR is homologous to the 50-kDa metalloproteinases secreted by *Serratia marcescens* and *Erwinia chrysanthemi* [10]. Alkaline protease degrades casein and gelatin, and migrates in zymography gels as a protein of approximately 56 kDa [11]. One group of research has also identified pseudomonal alkaline protease importance, and revealed that mutant strains that are unable to produce alkaline protease were unable to cause infection, whereas intact parent strains enhance extensive keratitis, virulence was regenerate to the "enzyme – deficient strains" by injection of small amount of alkaline protease in to the cornea [7, 12].
Interleukin-2 (IL-2) signals influence various lymphocyte subsets during differentiation, immune responses and homeostasis, stimulation with IL-2 is crucial for the maintenance of regulatory T (T Reg)) cells and for the differentiation of CD4 (+) T cells into defined effector T cell subsets following antigen-mediated activation. For CD8 (+) T cells, IL-2 signals

optimize both effector T cell generation and differentiation into memory cells. IL-2 is presented in soluble form or bound to dendritic cells and the extracellular matrix. Use of IL-2 - either alone or in complex with particular neutralizing IL-2-specific antibodies - can amplify CD8 (+) T cell responses or induce the expansion of the T (Reg) cell population, thus favoring either immune stimulation or suppression [13]. The present study was performed to determine and evaluate the inhibitory effect of crude bacterial suspension and pseudomonas alkaline protease enzyme on the rabbit interleukin - 2 following bacterial keratitis.

2. Materials and Methods

2.1 Animals

Eight apparently healthy male rabbits of six months of age and 3 kilo weight were sold from Vaccine and Sera Institute in Baghdad and kept in the animal house of the College of Biotechnology at the Al- Nahrain University for one month (April) to take care of them for the next steps of the research work.

2.2 Specimens collection

This study occurs during the period between April 2016 into January 2017 in the College of Veterinary Medicine / University of Baghdad. Eight serum samples were collected by withdrawn 5 ml of blood from male rabbit's veins following disinfection of the skin by using ethyl alcohol at 95% for each one. All blood samples centrifuged at 20000 rpm for 15 minutes for separating the serum from the blood [14]. Then all collected serum were preserved by freezing at 20- until demonstration of IL2 by enzyme linked immune sorbent assay (ELISA) in the next step.

2.3 *Pseudomonas aeruginosa* isolation

Approximately 6 suspected bacterial isolates of *P. aeruginosa* were obtained by swabbing from ear doges suffering from otitis media from Adan Veterinary Hospital in Baghdad. All swabs were streaked onto cultural media which included the Blood agar and MacConkey agar plates then incubated at 37 °C for 24 to 48 hours depending on the nature of the sample.

2.4 Bacterial identification

The bacterial isolates were identified and characterized as *P. aeruginsa* by using a combination of colonial morphology, Gram stain characteristics and biochemical reactions such as oxidase and catalase tests, and then confirmed by inoculated them on the selective and differential media for *P. aeruginosa* called citromid agar [15]. One confirmed isolate of *P. aeruginosa* was utilized in the study for initiation the infection for one group of rabbits (a crude bacterial suspension treatment group).

2.5 Preparation of bacterial inoculums

The bacterial inoculums was prepared from a pure fresh culture of citromid agar media and aseptically few bacterial colonies were transferred by disposable lope into 5.0 ml of sterile Brain heart infusion broth, then incubated at 37 Cº for 24 hours.

2.6 Bacterial and enzyme injection

A freshly prepared crude bacterial suspension of *P. aeruginosa* was injected intrastromly in the cornea of the first group a crude bacterial suspension treatment group) that's consist of four rabbits at a dosage of 500µl in a form 5 drops and by using sterile insulin syringe and needle cage no. 3 and leaving them for 5 hours for appearing signs and symptoms of infection like lacrimation, redness of eye, and inflammation. At the same time the remaining four rabbits (enzyme treatment group) were injected intrastromly with pure pseudomonas alkaline protease enzyme by the same dosage, time, and by the same way of injection of bacterial suspension.

2.7 Kit and enzyme

i) Rabbit Interleukin 2 (IL2) ELISA kit: (YH Biosearch Laboratory) catalogue number (YHB0071Rb). This kit uses enzyme- linked immune sorbent assay based on biotin double antibody sandwich technology to assay rabbit IL2. Add IL2 to wells that are pre-coated with IL2 monoclonal antibody and then incubate.

ii) Alkaline protease solution: 3 ml at a concentration 500 units ±10% **(**Promega, USA) catalogue number (A1441).

2.8 Statistical analysis

According to standards concentrations and corresponding OD values, the linear regression equation of the standard curve were calculated. Then according to the OD value of samples, the concentration of the corresponding sample calculated statistically by using SPSS program.

3. Results

Fig. 1 and table 1 showed three types of regression equations tested for the using of OD values in the predicting the concentration. The quadratic and cubic equations were equally superior to linear model in predicting the concentration as showed by the higher value of determination coefficient (R^2). The quadratic model was chosen in favor of cubic model because of its simplicity.

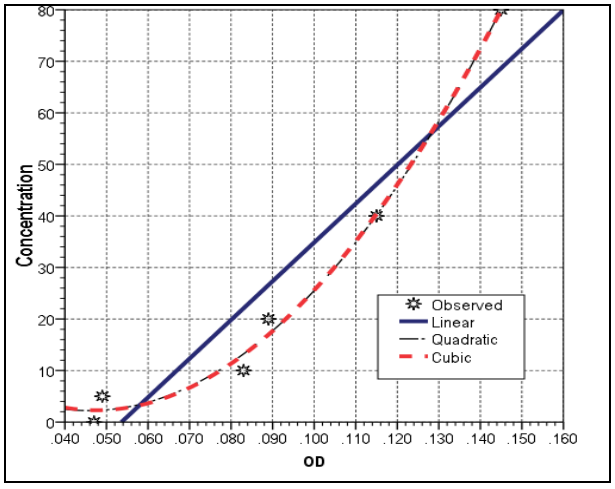

Fig 1: Scattered diagram with fitted linear, quadratic, and cubic regression for the concentration over OD values using eight calibration samples wells and six standard wells of IL- 2 that's examined by ELIZA.

Table 1: Three types of regression and determination coefficient values

	R^2	P
Linear model	0.900	0.004
Quadratic model	0.993	0.001
Cubic model	0.993	0.01

Concentration = 18.66-(719.97 x OD) + (7884.73 x OD^2).

Among the bacterial suspension treatment group the serum IL2 significantly reduced by a mean of 14 ug/dl after

treatment. The reductions ranged between a minimum of 7.4 ug/dl to a maximum reduction of 22.1 ug/dl. The treatment effect in this group was evaluated as a strong effect (Cohen's d >0.8) (table 2, fig. 2,3 and 4).

Also table 2 and fig. 2,3, and 4 it has been shown that the enzyme treatment group the serum IL2 showed no statistically significant departure from a mean change of zero after treatment. The changes after treatment ranged between reductions of as high as 57 ug/dl to an increase of as high as 32 ug/dl. The treatment effect in this group was evaluated as a very weak effect (Cohen's d<0.3).

Table 2: Changes in serum IL2 (ug/dl) after two types of corneal treatment.

	Baseline	After treatment	Changes after treatment	P (paired t-test)	Cohen's d
Bacterial suspension				0.02	-1.67
Range	(20.7 to 43.5)	(8.6 to 21.4)	(-22.1 to -7.4)		
Mean	30.1	15.4	-14.7		
SD	11.1	5.7	6.2		
SE	5.53	2.83	3.08		
N	4	4	4		
Enzyme				0.79[NS]	0.28
Range	(12.6 to 59.7)	(2.7 to 50.6)	(-57 to 32)		
Mean	27.3	33.5	6.2		
SD	22.1	21.6	42.3		
SE	11.06	10.78	21.16		
N	4	4	4		
	0.83[NS]		0.37[NS]		

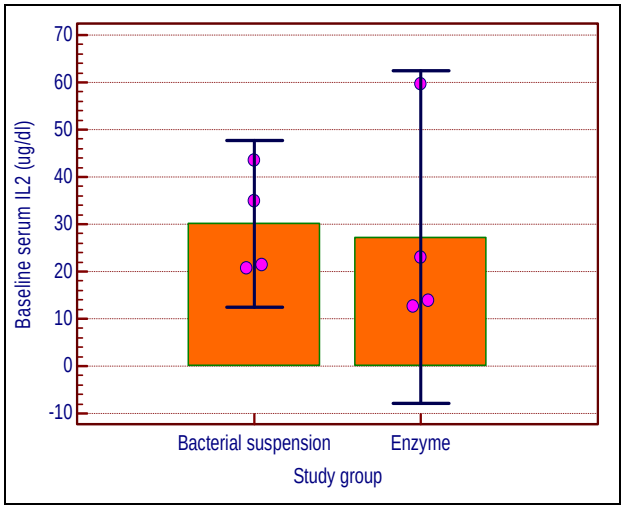

Fig. 2: Dot diagrams with error bars showing the difference in mean (with its 95% confidence interval) serum IL2 at baseline between the two treatment groups.

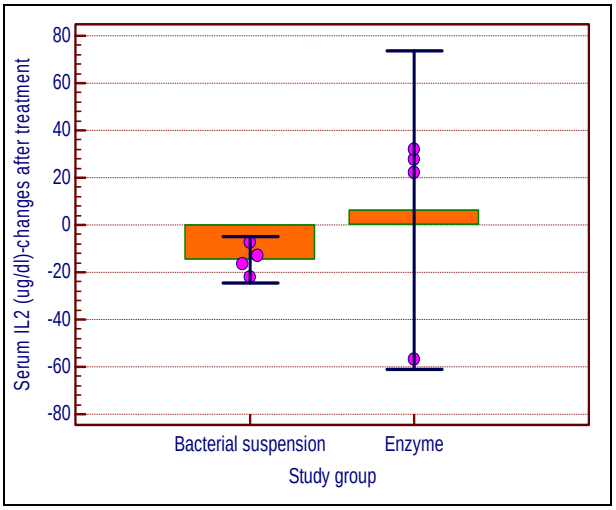

Fig. 3: Dot diagrams with error bars showing the difference in mean (with its 95% confidence interval) change after treatment for serum IL2 between the two treatment groups.

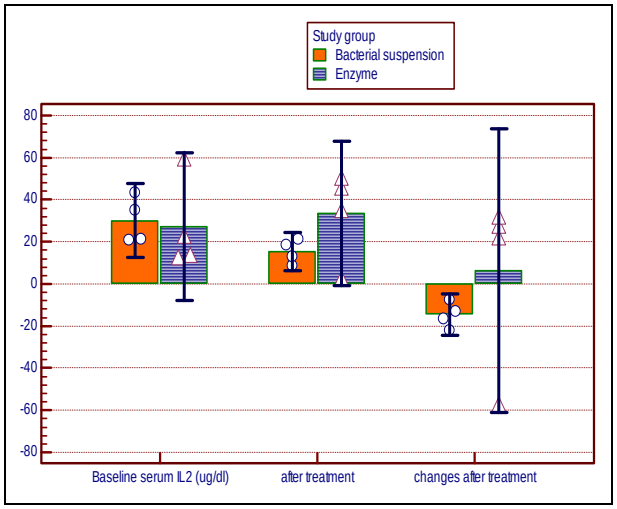

Fig. 4: Dot diagrams with error bars showing the difference in mean (with its 95% confidence interval) serum IL2 between the two treatment groups (at baseline, after treatment and changes after treatment).

4. Discussion

Different strains of *P. aeruginosa* secrete several extracellular proteolytic enzymes that have been implicated as virulence factors. They include protease IV, alkaline protease (aeruginolysin), and two elastases, LasA (staphylolysin) and LasB (pseudolysin) [11]. APR is implicated in hydrolysis of many biologically important proteins including cytokines [16], complement factors [17], laminin [18], matrix metalloproteinases [19], human γ-interferon and tumor necrosis factor-α [16].The present study showed that the serum IL2 level among crude bacterial suspension treatment group (following initiation of infection) was significantly reduced by a mean of 14 ug/dl after treatment. In comparison with serum IL2 level among the alkaline protease enzyme treatment group where showed no statistically significant departure from a mean change of zero after treatment. This give a strong guide for the potency and effectiveness of bacterial agent in the degradation and destruction of immune response elements like IL2 at a higher level in contrast to virulence factor of *P. aeruginosa* like alkaline protease which give a weak potency

in the degradation of the serum IL2 although the bacterial suspension that's utilized in this study was a crude while the alkaline protease was purified at 50%. The present study results proved an important fact that alkaline protease enzyme is considered as a weak virulence factor in the pathology of bacterial keratitis and can't enhanced damage of the cornea by alone represented by weak performance on the serum IL2 of the eye rabbits. This result is in consisted with previous study done by [9] that's suggested the production of elastase during *P. aeruginosa* keratitis enhances ocular pathology, whereas alkaline protease production contributes to limited corneal erosion. Another study supported the present study suggestion that the corneal destruction seen with *P. aeruginosa* infections may result from enhanced expression of matrix metalloproteinases (MMPs) by corneal stromal cells stimulated with pseudomonal exoproteases and proinflammatory cytokines and the proteolytic activation of MMPs by pseudomonal elastase [20].

The role of *P. aeruginosa* alkaline protease in keratitis is still controversial previous studies reported that strains of *P. aeruginosa* PA103 deficient in alkaline protease production were not able to establish corneal infections and could not colonize traumatized cornea [21]. Afterwards an *in vivo* infection study in mice showed that active alkaline protease was present in corneal tissues [22]. In contrast Pillar and coworkers constructed alkaline protease-deficient mutants of *P. aeruginosa* to analyze the role of this protease in corneal infection and they were able to show that the alkaline protease has no influence on the ocular virulence of *P. aeruginosa* [23]. Because of these results it was concluded that alkaline protease is not essential for *P. aeruginosa* keratitis contrary to the former opinion.

The role of secreted proteases in the pathogenesis of corneal disease was further examined by using proteases mutant strains of *P. aeruginosa* [24]. This study showed that the three proteases alkaline protease, LasA, and LasB are not essential for the establishment of the ocular virulence of *P. aeruginosa*. However, it was demonstrated that the alkaline protease seems to be a crucial mediator of virulence. This function of the alkaline protease depends on the location of *P. aeruginosa* within the cornea and on the presence of simultaneous elastolytic activity [24].

5. Conclusion

The present concluded that the bacterial agent of P. aeruginosa as a crude suspension exhibited a higher effect for lowering and degradation of serum IL2 in comparison to the purified pseudomonas alkaline protease enzyme which give a weak effect in the degradation of serum IL2 and this proved that the alkaline protease enzyme can't enhanced bacterial keratitis or corneal erosion by alone unless there are other proteases enzymes especially pseudomonal elastase is available.

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