

Mice Corneal Damage Mediated by *Pseudomonas aeruginosa* Extracellular Proteases*

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الملخص

تعد انزيمات الـ Proteases الخارج خلوية من عوامل الضراوة المهمة للزوائف الزنجارية *Pseudomonas aeruginosa* والتي تلعب دوراً مهماً في الاصابات المرضية لقرنية العين . كانت النسبة المئوية لانتاج هذه الانزيمات من قبل هذه الجرثومة المعزولة من حالات مرضية مختلفة والتي أظهرت تلفاً لقرنية العين في الفئران 12 % من مجموع 25 عزلة تم الحصول عليها من مستشفى الزهراوي في الموصل .

أدى حقن المستخلصات الخام لهذه الانزيمات في قرنية عين الفئران الرضعية بعمر 5 أيام الى موت معظمها خلال 24 ساعة. وادى حقن نفس المستخلصات في قرنية عين الفئران بعمر 30 يوماً تأثيراً بالغاً ظهر عياناً كبقعة بيضاء في موقع الحقن خلال 4-5 أيام.

أظهر حقن الانزيمات المنقاة جزيئاً نفس التأثيرات العيانية ولكن خلال 24 ساعة فقط مما يؤكد دور انزيمات proteases كعوامل مهمة من عوامل ضراوة هذه الجراثيم في اصابات قرنية العين .

ABSTRACT

Extra cellular Proteases of *Pseudomonas aeruginosa* had been considered to be one of the important virulence factors in corneal infections.

Production of these enzymes from strains that elicited corneal damages in mice were detected in approximately 12 % of 25 isolates obtained from Al- Zahrawii hospital in Mosul.

Intra -corneal injection of neonates 5 days old with the crude proteases preparation cause death most of them within 24 h .

The injection of these preparation intra corneally into 30 days old mice elicited severe damages that grossly appeared as a white spot within 4-5 days at the site of injection. While the injection of partially purified proteases produced the same corneal damage which was identified grossly within 24h only. This revealed the importance of these enzymes as a virulence factor for these bacteria in corneal infection.

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Introduction

The gram-negative bacillus *Pseudomonas aeruginosa* is a significant cause of bacterial keratitis in patients who use extended wear contact lenses (1). Infection with this bacterial results in a particularly rapid and destructive disease that if untreated can result in corneal perforation and loss of the eye in 2 to 4 days(2).

In the eye , mucus is the predominant component of the tear film , a three layered film on the surface of the eye forming the first barrier between the outside environment and the underlying corneal epithelial cells (3). It is constituted primarily of secretory mucin secreted from conjunctival goblets (4). In general , the mucin layer has a protective role in overlying the epithelial cells. Invitro studies have documented that pre incubation of microbes with mucin decreased their subsequent adhesion to epithelial cells (5 , 6). *Pseudomonas aeruginosa* Produces a variety of extra cellular factors that are potentially cause damaging to ocular tissues , the organism is capable of producing a number of proteases (Alkaline proteases staphylolytic protease , elastase , and protease IV) (7).

Binding of *P. aeruginosa* to the cornea is stimulated by protease digestion of the corneal surface Protease digestion could un mask receptor or induced synthesis of additional receptors (8).

Mucin degradation by bacterial proteases may also be a necessary for bacteria to use mucin as nutrient source (9). There is an important interaction between *P. aeruginosa* and mucin which point toward the ability of these strains to produce proteases that degrade mucin. Non protease producing strains did not appreciably degrade mucin (9).

Pseudomonas – proteases elicit severe corneal damage by causing the loss of the corneal proteoglycan ground substance , thus resulting in dispersal of undamaged collagen fibrils weakening of the corneal stroma (10).

The purpose of this research is to reveal the important role of the extracellular proteases in the corneal damage caused by *Pseudomonas aeruginosa* .

Material and Methods

Bacterial strains and culture condition :

25 strains of *Pseudomonas aeruginosa* were obtained from Al-Zahrawii hospital. Bacterial strains were cultivated in brain heart infusion broth , 100 ml flasks containing 25 ml of broth were inoculated with 0.1 ml of stationary- phase culture of the bacterium and incubated for 24 h at 37°C.

Preparation of crud and partially purified proteases

The procedure was done according to Krecer and Gray at 4°C in brief. The culture supernatant fluid of flasks culture after incubation at

37°C for 24 were obtained by centrifugation (at 9000g × 15 min) the resultant designated as (crude enzyme).

partial purification :

Ammonium sulfate was added slowly to the pooled culture supernatant fluid with gentle stirring to a final concentration of 90 % saturation. After one day the precipitate was recovered by stirring 16 g of cellulose powder in to the preparation and the mixture was filtered through a coarse-porosity sintered glass filter. The precipitate (Cake) was extracted twice with sufficient 0.1 M ammonium bicarbonate (AB) pH 7.8 insoluble residue was removed from the extract by centrifugation and membrane filtration (0.45 Mm pore size). The extract was dialyzed for 1 day against 0.1 μ (AB) the resultant designated as partially purified enzyme.

Detection of Proteases activity

Proteases activities were detected by determination of gelatin liquefaction according to (11) in which 0.2 ml enzyme preparation (crude and partially purified) was added to 2 ml of 12 % gelatin poured in tubes control for this test was done by adding 0.2 ml of D.W instead of enzyme preparation.

The test and control tubes were incubated in water bath at 55°C for 30 min and then cooled in 25°C water bath for 1 h. A positive reaction was scored if the gelatin remains liquid after removal from the water bath.

Production of corneal damage by enzyme preparation

In the first experiment 5 days neonates BALB-C mouse (10 mice) were injected intracorneally with 0.1 ml of crude enzyme preparation while the eyelids remained fused, care was taken not to wound the ocular surface by gently elevating the eyelid away from the corneal surface with a small forceps before injection.

Later 30-days mouse (25 mice) were also injected intracorneally with 0.1 ml of crude enzyme preparation and corneal damages were observed daily.

Partially purified preparation were injected also into adult mice. All mice in the experiments were anesthetized before injection of the enzyme preparation (crude and partially purified). Controls received sterile saline in a similar volume and manner as the experimental one.

Result and Discussion

Three cornea – virulent of twenty five strains of *Pseudomonas aeruginosa* were identified for the production of extracellular proteases that elicited severe corneal damages in mice and exhibited positive result in gelatin test (Fig 1).

In an initial experiment , an attempt was made to produce infections by injecting infant mice with crude protease preparations. Most of them died within 24 hrs.

Corneal damages occurred at a rate of 3/25 (12%) after 4-5 days of injected mice (30 days old) with the same preparation. A first damages appeared as opacification of the cornea at the site of injection then , turned to a white spot within 4-5 days as shown in (Fig 3). On the other hand , partially purified protease preparation elicited these damages in less than 24 hrs. after injection , as shown in (Fig 4). None of the injected mice died possibly due to more developed immune system compared to infants. These results are supported by earlier studies of Berk et al who concluded that the ocular surface of infant mice is more permeable than that of adults. This permeability may be due to incomplete maturation of the cornea, and Pei and Rhodin who considered that the murine corneal epithelium is not morphologically mature and does not reach full growth only at about 14 days post partum. Hazlett et al also has found that the precorneal tear film is not morphologically demonstrable in mice until about the time of eyelid opening. Thus an immature animal lacking a demonstrable tear film possesses an ocular surface with a deficient epithelial barrier which might be ineffective in preventing toxin penetration , once the toxin penetrates the ocular epithelium it may then gain access to the general circulation and cause lethality in 5 and 10 day old mice. Older animals in which the epithelium and associated tear film are fully developed may be able to sequester or slow penetration by the toxin and eventually inactivate it in situ (15). The results of this study also revealed the important role of extracellular proteases produced by three *Pseudomonas aeruginosa* strains elicited severe corneal damages in mouse within less than 24 h.

These results agree with other studies such as Aristotel and Willeox who concluded that protease activity was the prime mediator of mucin degradation by *P. aeruginosa* , only strains producing appreciable level of protease activity were able to deplete mucin, while clinical isolates producing low level of protease were unable to degrade mucin. Mucin degradation by protease action weakens protective mucosal coat and facilitates bacterial penetration of underlying epithelial cells. The proteolytic hydrolysis of mucin by certain *P. aeruginosa* isolates observed is set to be a factor that may influence the infectivity of that strain in the eye. Indeed the removal

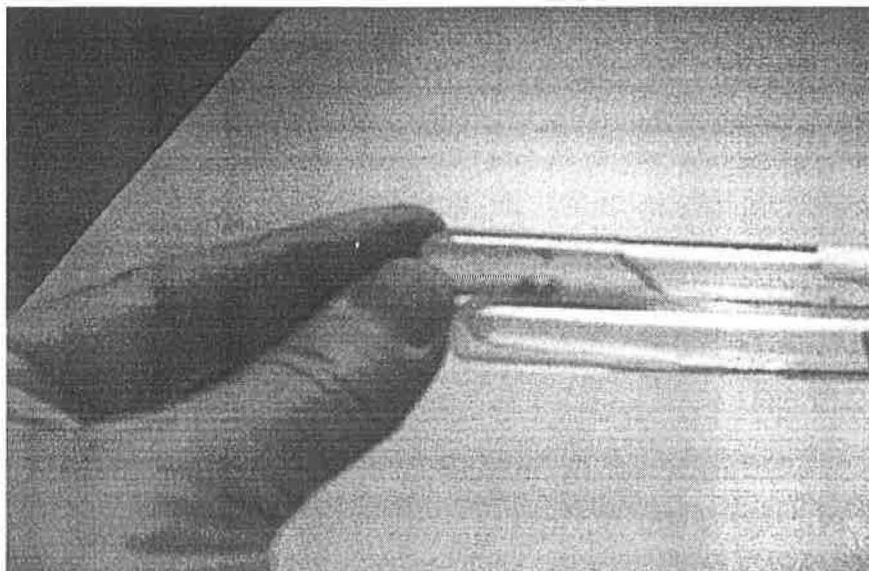


Fig1 : Detection for proteases activity by Liquification of gelatin, positive reaction (Lower tube). Negative reaction (upper tube) didn't liquefies gelatin.

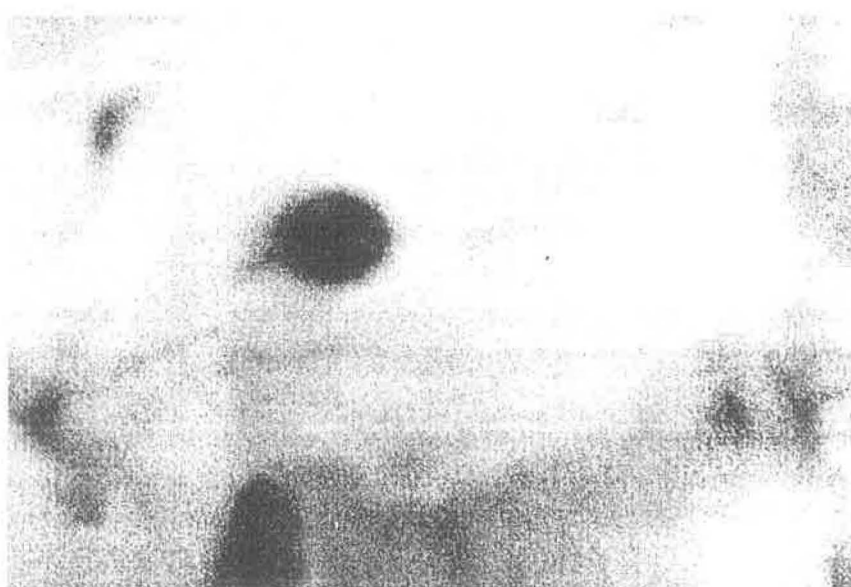


Fig 2 : Control cornea of 30 days old mouse that injected with 0.1 ml normal salin.

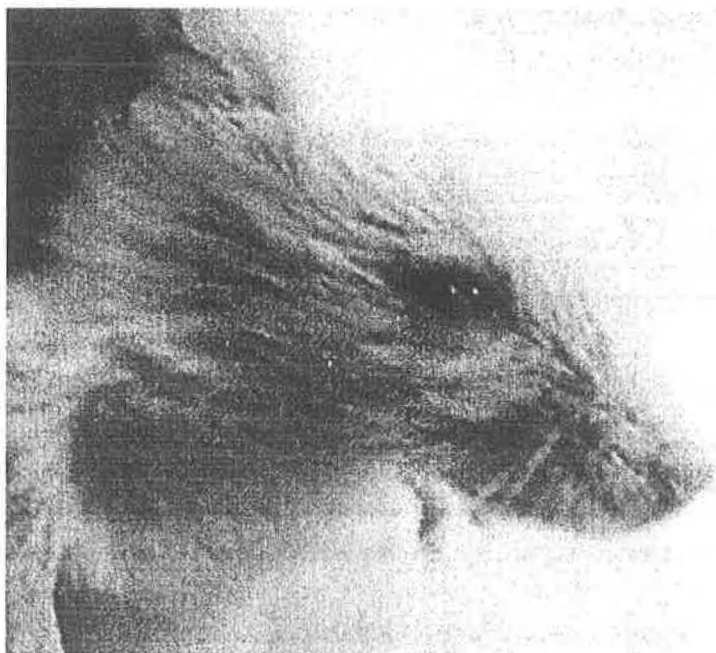


Fig 3 : Corneal damages of 30 days old mouse produced after 4-5 days postinjection with 0.1 of crude *P. aeruginosa* proteases.



Fig 4 : Corneal damages of 30 days old mouse produced after less than 24h postinjection with 0.1 of partially purified *P. aeruginosa* proteases.

of ocular mucus from the cornea in vitro resulted in increased adhesion of *P. aeruginosa* to the corneal surface (16). Others (10) and (17) described that protease – induced structural damage observed by light and electron microscopy during experimental *P. aeruginosa* keratitis supports the idea that the ability of *P. aeruginosa* to elicit rapid liquefaction necrosis of the cornea is related at least in part , to the invivo production by the

bacterium of extracellular cornea – damaging proteases. This idea is further supported by the reported observation of (18) that two non proteolytic strains of *P. aeruginosa* produced much less severe experimental corneal diseases than strongly proteolytic strains.

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