

## Study of *Pseudomonas aeruginosa* Proteases Enzymes in Corneal Ulceration by Using Real - Time PCR

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### ABSTRACT:

#### BACKGROUND:

*Pseudomonas aeruginosa* (*P. aeruginosa*) is the most frequently isolated bacteria in clinical cases of ulcerative keratitis. *P. aeruginosa* produces several proteases and toxins, the best characterized being exotoxin, elastase, alkaline protease and IV. These are all important factors in the establishment of bacterial infection and the amount of damage caused by the infection to the cornea.

#### OBJECTIVE:

The aim of the present study was to study the role of *P. aeruginosa* proteases ( elastase ( LasB ), LasA, Alkaline protease, and protease IV ) in corneal ulceration by using Real - time PCR.

#### METHODS :

One hundred - twenty clinical samples (corneal scraping) were collected from patients suspected with bacterial keratitis presenting to Ibn –Alhaytham Teaching Hospital from May 2013 until November 2013. Methods for isolation and identifying *P. aeruginosa* based upon culture coupled with biochemical tests and confirmed by new technique called Vitek 2 compact system. The role of proteases enzymes ( elastase ( LasB), LasA, alkaline protease and protease IV ) of *P. aeruginosa* in the corneal ulceration was studied by Real – time PCR.

#### RESULTS :

Real time analysis demonstrated that three bacterial isolates of *P. aeruginosa* were possessed elastase gene (11.5%), one bacterial isolate was harbored LasA gene (3.8%), twenty bacterial isolates were possessed protease IV gene ( 76.9%) , and all bacterial isolates were possessed alkaline protease gene (100%).

#### CONCLUSION:

The presence of the alkaline protease and protease IV genes in almost bacterial isolates of *P. aeruginosa* improved the fact that these enzymes were not only tissue damaging but also very important colonizer agents to cornea.

**KEY WORDS:** *Pseudomonas aeruginosa* proteases, Corneal ulceration, Real – time PCR.

### INTRODUCTION:

Bacterial keratitis is an infectious disease of the cornea caused by bacteria. It is a serious ocular infectious disease that leads to severe visual disability. Many patients have a poor clinical outcome if aggressive and appropriate therapy is not promptly initiated <sup>(1)</sup>. Bacterial keratitis is rare in the absence of predisposing factors. Up to year 200, most cases of bacterial keratitis were associated with ocular trauma or ocular surface diseases. However, the widespread use of contact lenses has dramatically increased the incidence of contact lens related keratitis <sup>(2,3)</sup>. *P. aeruginosa*, is

the most frequently isolated organisms in clinical cases of ulcerative keratitis. Its commonly associated with various predisposing factors mainly ocular surface disease, ocular trauma, ocular surgery, and contact lens wear which are a major risk factors for the development of microbial keratitis <sup>(4,5,6,7)</sup>. The resultant pathologic damage in *Pseudomonas* keratitis is depending on a production of a broad spectrum of extracellular enzymes including proteases enzymes. Proteases enzymes such as elastase, LasA (staphylolysin), LasB, alkaline protease, and protease IV enzyme have been characterized in many clinical settings, including corneal ulcer <sup>(8)</sup>. Bacterial culture and smear examination using corneal scrapings is the conventional method to detect causative pathogens of corneal ulceration. However, bacterial culture is time consuming and results of

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smear examination depend on the laboratory technician's skill <sup>(9)</sup>. Therefore, a fast and accurate diagnostic method is highly desirable <sup>(10)</sup>. In recent years, Real – time PCR has been widely used for clinical bacterium, and virus - specific detection and various technologies have been developed for the PCR assay <sup>(11, 12,13)</sup>. Quantitative

PCR ( Real – time PCR ) and DNA microarray are modern methodologies for studying gene expression and the future of qPCR remains bright as the technology becomes more rapid, cost-effective, easier to use, and capable of higher throughput. <sup>(14,15)</sup>. The Real - time PCR assay can simultaneously detect and quantitate bacterial and fungal pathogens in patients with corneal ulcer. Real time PCR can be a fast diagnostic tool and may be useful as an adjunct to identify potential pathogens <sup>(16)</sup>.

### SUBJECTS AND METHODS:

#### Specimen collection

One - hundred and twenty patients with suspected microbial keratitis (corneal ulcer) presenting to Ibn Al-Haytham Teaching Eye Hospital from May 2013 until November 2013 were included in this study. Corneal scraping was done by an ophthalmologist using a sterile bent-tipped needle.

#### Identification :

Bacterial identification was performed by using biochemical tests. These include Gram reaction, growth characteristics, and other test methods such as oxidase and catalase tests <sup>(17)</sup>.

#### Conformation :

By Vitek 2 Compact system (The mentioned tests; along with the Vitek Compact 2 System confirmed that 26(21)isolates belonged to *Pseudomonas aeruginosa*).

### DNA extraction and PCR analysis of Protease enzymes of *Pseudomonas aeruginosa*

#### Isolating Genomic DNA from Gram Negative Bacteria

Genomic DNA was prepared for twenty six bacterial isolates chosen according to Vitek 2 Compact System results. In this study we used Wizard Genomic DNA Purification Kit for extraction and purification DNA from Gram negative bacteria.

1. One millimeter of an overnight cultures of *P.aeruginosa* isolates cultivated aerobically on Lauria broth were added to a 1.5ml microcentrifuge tubes.
2. Centrifuged at 13000–16000 × g for 2 minutes to pellet the cells. The supernatant were removing.

3. Six hundred µl of Nuclei Lysis Solution was added. Gently pipette until the cells are resuspended.
4. Then the suspension was Incubated at 80°C for 5 min. to lyses the cells; then cooled to room temperature.
5. Three µl of RNase solution was added to the cell lysate. Then the tubes were invert 2–5 times to mix and were incubated at 37°C for 15–60 minute . Cooled the sample to room temperature.
6. Two hundred µl of protein precipitation solution was added to the RNase-treated cell lysate. Vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate.
7. The samples were incubated on ice for 5 min.
8. Centrifuged at 13000–16.000 × g for 3 min.
9. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol.
10. Gently mixed by inversion until the thread-like strands of DNA form a visible mass.
11. Centrifuged at 13000–16000 × g for 2 minutes.
12. Carefully pour off the supernatant and drain the tube on clean absorbent paper. Add 600µl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet.
13. Centrifuged at 13,000–16,000 × g for 2 min. Carefully aspirate the ethanol.
14. The tubes were drained on clean absorbent paper and allow the pellet to air-dry for 10–15 min.
15. One hundred µl of DNA Rehydration solution was added to the tube and rehydrate the DNA by incubating at 65°C for 1 h. Periodically the solution was mixed by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C.
16. The DNA was stored at 2–8°C.

### Real-Time PCR analysis of proteases genes of *Pseudomonas aeruginosa*

#### Primers

The sequence of the primers used for detection of genes coding for these *P.aeruginosa* proteases were included:

- 5'-  
TGGGAAGGCCGGATCCACGCCGAGGCG(T  
CF),  
5'-  
'CAGCCGGGGACCGAATTCCTTAACGCGC

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T (TCR) yielding a product of 942 bp for elastase (LasB) gene which designed on previously published studies (18).

5'-GCCGAGGCCGCGTAGAGGATGTC (TCF)

5'-GCCGAGGCCGCGTAGAGGATGTC (TCR), yielding 993 bp product for alkaline

### Detection of proteases genes by Real - time PCR technique:

Polymerase chain reaction amplifications of proteases enzymes (Elastase (LasB), LasA, alkaline protease and protease IV) were performed by using thermal cycler. Each gene was amplified separately.

protease gene which designed on previously published studies (18).

5'-TATTTCCGCCGACTCCCTGTA(TCF), 5'-GAATAGACGCCGCTAGAAATC (TCR), yielding a 353 bp product for (IV)protease

described by Cabellero and colleagues (9).

5'-TGTTCTTCCAGGAGCGTGG-3 (TCR), 3'-TCGCAAATCACATCGAC-5', yielding 424 bp product for LasA protease (19)

Program the thermal cycler as per the manufacturer's instructions using the following guidelines (Table 1):

Table 1: Guidelines of thermal cycler.

Components	# Cycles	Standard Cycling Program
Hot-Start Activation	1	95°C for 2 min
Denaturation	40	95°C for 15 min
Annealing	40	60°C for 15 sec.
Dissociation	1	60-95°C

The data was designated and collected during the annealing step of each cycle. Statistical Analysis: The Statistical Analysis System -SAS (2010) was used to analysis of data to effect of difference factors in study percentage. The chi-square test was used to significant compare between percentage at 0.05 and 0.01 probability.

### RESULTS :

The resultant isolates identified as *P. aeruginosa* were 26 (21.6%), four set of primers pairs were used in this study targeted the proteases enzymes genes (elastase (LasB), LasA, alkaline protease, and IV). Real- time analysis of our present study has been demonstrated that three bacterial isolates of *P. aeruginosa* were possessed elastase gene

(LasB) and named as ( P3, P23,P24) respectively at a percentage (11.5%) ( Figure 1) ,only one isolate were harbored LasA gene and named as (P85) at a percentage (3.8%) ( Figure 2), also our current study was showed that all bacterial isolates were possessed alkaline protease (P1, P3, P14 , P16, 21, 22, 23, 24, 26 , 31, 32, 33, 34, 35, 41, 43, 59, 63, 69, 81, 82, 83, 85, 88, 107, 108) respectively at a percentage (100%) ( Figure 3) and twenty bacterial isolates of *P. aeruginosa* were possessed protease IV that's named as ( P1, P3, P14 , P16, 21, 22, 23, 24, 26 , 31, 32, 35, 41, 43, 59, 69, 81, 82, 88, 108) respectively at a percentage ( 76.9%) (Figure 4).

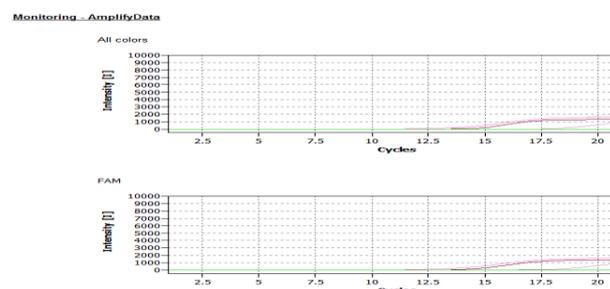


Figure 1: Real - time analysis of *Pseudomonas aeruginosa* isolates were showed that only three isolates were harbored elastase gene (LasB) and were named as ( P3, P23,P24) respectively.

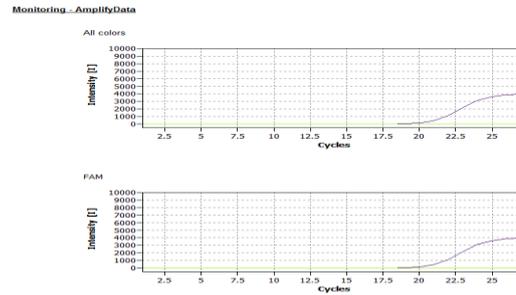


Figure 2: Real - time analysis of *Pseudomonas aeruginosa* isolates show that only one isolate) harbored LasA gene and was named as (P85).

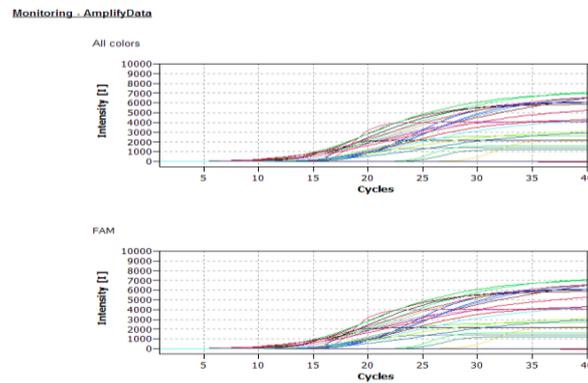


Figure 3: Real - time PCR analysis of *Pseudomonas aeruginosa* isolates was showed that all bacterial isolates were harbored alkaline protease and were named as ( P1, P3, P14 , P16, 21, 22, 23, 24, 26 , 31, 32, 33, 34, 35, 41, 43, 59, 63, 69, 81, 82, 83, 85, 88, 107, 108)

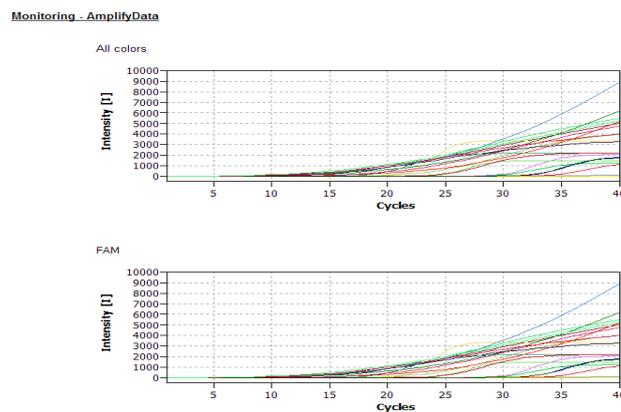


Figure 4: Real - time PCR analysis of *Pseudomonas aeruginosa* isolates was showed that all bacterial isolates were harbored alkaline protease and were named as ( P1, P3, P14 , P16, 21, 22, 23, 24, 26 , 31, 32, 35, 41, 43, 59, 69, 81, 82, 88, 108)

Our results also indicated that three bacterial isolates of *P. aeruginosa* (p3, p23,24) (11.5%) were harbored more than one gene of different proteases enzymes( elastase, alkaline protease, and protease IV). On other hand our

### DISCUSSION:

The resultant pathologic damage in *Pseudomonas* keratitis is depending on a production of a broad spectrum of extracellular enzymes including proteases enzymes. Proteases enzymes such as elastase, LasA (staphylolysin), elastase (LasB), alkaline protease, and IV enzyme have been characterized in many clinical settings, including corneal ulcer<sup>(8)</sup>. Real- time analysis of our present study has been demonstrated that three bacterial isolates of *P. aeruginosa* were possessed elastase gene (LasB) at a percentage (11.5%) These results are not consistent with previous studies which indicated that the bacterial isolates of *P. aeruginosa* isolated from bacterial keratitis were at high productivity of the enzyme and was estimated at (51%), and these isolates show high efficacy for it<sup>(20)</sup>. In another study conducted on 145 bacterial isolates (in the U.S.) isolated from different clinical sources and was included bacterial keratitis. It was founded that all isolates harbored the gene responsible for production of elastase ( LasB). However, four isolates were failed to expressed elastase gene (LasB)<sup>(21)</sup>. The difference between our result and previous studies may be explained by many reasons : first reason is due to small size of our sample collected in comparison with these studies, second the usage of non- genetic conventional methods by other researcher to detect elastase (LasB) production such as Elastin - nutrient agar which considered as one of the qualitative methods used for detection of enzyme activity, this method does not expressed the LasA gene of bacterial isolate in comparison to Real - time PCR technique, and third reason discussed by genetic study conducted on many bacterial isolates isolated from different clinical sources, this study suggested that most of the bacterial isolates that causes bacterial keratitis were genetically variable from other bacterial isolates isolated from different infections<sup>(22)</sup>. Another study was carried out on 61 bacterial isolates isolated from bacterial keratitis which indicated that the *P. aeruginosa* does not appear uniformly in the production of

results showed that one bacterial isolates (p85)(3.8%) was harbored both of LasA and alkaline protease gene, and twenty bacterial isolates of *P. aeruginosa* (76.9%) were harbored alkaline protease and protease IV gene together.

virulence factor, as the part of these samples produced proteases enzymes ( Elastase, alkaline protease, LasA, and protease IV). These results indicated that the bacteria does not possess uniform in occurring the disease<sup>(23)</sup>. On the other hand our present study was showed that only one isolate harbor LasA gene at percentage (3.8%) and named as (P85), These result is disagree with previous studies, where the study was carried out by Kadhim, (2008)<sup>(20)</sup>, and conducted on 17(8.5%) bacterial isolates of *P. aeruginosa* showed that all bacterial isolates of *P. aeruginosa* isolated from corneal ulcer were harbored the gene responsible for production of LasA enzyme. Another study showed that 40% of bacterial isolate isolated from corneal ulcer harbor LasA gene<sup>(21)</sup>. The difference between our result and previous studies perhaps due to usage of non - genetic methods to demonstrate the presence of LasA gene which depends on the usage of LB and measured what is called qualitative activity of enzyme, this method does not expressed the LasA gene of bacterial isolate in comparison to Real - time technique, and our study may reflect the fact that either the LasA enzyme is not major virulence factor in the *Pseudomonas aeruginosa* keratitis or the bacterial isolates of *P. aeruginosa* show genetic variation in occurring the disease. Also Real - time PCR analysis was showed that all bacterial isolates of *P. aeruginosa* were harbored alkaline protease genes, at a percentage (100%). These results are in agreement with study conducted by Kadhim, (2008)<sup>(20)</sup> where reported that all isolate of *P. aeruginosa* of corneal ulcer and wound have the ability to produce the alkaline protease. Also another study agree with our results, where indicated that all bacterial isolates isolated from different clinical source have the highest activity of enzyme<sup>(23)</sup>. Our results were supported with what stated about the alkaline protease of *Pseudomonas aeruginosa* it is not only tissue damaging but also its very important colonizer agent and has been associated with bacterial virulence and is known to interfere with complement-mediated lysis of erythrocyte. Mutants deficient in alkaline protease production could not colonize traumatized cornea and did not produce the corneal damage characteristic of infection by the parental strain<sup>(25)</sup>. The presence of the corneal ulcer and tissue damage of our patients give us very strong evidence for the presence of the alkaline protease genes in all our

bacterial isolates. However, these results were in agreement with another study where indicated that 25% of bacterial isolates have the ability to produce the enzyme<sup>(26)</sup>. The difference perhaps due to the diversity in the isolation sources that has depended on it, as it has been isolated from different clinical sources (urine, burns, ear, wounds, blood, and pus). Finally Real-time analysis of all bacterial isolates of *P. aeruginosa* were showed that twenty bacterial isolates harbored protease IV at a percentage (76.9%). These results are in agreement with previous studies which stated that protease IV was secreted by most *P. aeruginosa* isolates that causing microbial keratitis and in association with other proteases has a major role in corneal virulence<sup>(27,28)</sup>.

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