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ORIGINAL ARTICLE

Experimental Intrastromal Antibiotic Injection for Refractory Keratits Caused by Biofilm Forming *Pseudomonas Aeruginosa*

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Key words:

Intrastromal Antibiotic Injection, Refractory Keratitis, Biofilm, Pseudomonas

ABSTRACT

Background: *Refractory keratitis is a major ophthalmic concern, unresponsive patients to ordinary or fortified eye drops or even specific antibiotic drops according to the cultures taken from the lesions may necessitate therapeutic keratoplasty with poor prognosis. Biofilm is an important virulence mechanism for bacterial infections and is a major cause of antibiotic resistance. Treatment of biofilm requires delivering of higher antibiotic concentration than normal.* ***Objectives:*** *This study aims to evaluate the role of biofilm in formation of keratitis and to consider intrastromal antibiotic injection to deliver higher concentration of antibiotics to treat keratitis caused by biofilm forming pseudomonas.* ***Methodology:*** *We used 40 New Zealand white female rabbits in this prospective in-vivo experimental study. Rabbits were classified in to four groups: one eye (the right one) in all rabbits was used for the inoculation with the organism as a test eye while the left eye was used as a control. The right eye of group (1) was inoculated with biofilm forming Pseudomonas aeruginosa and treated with intrastromal ciprofloxacin injection, while, first eye of group (2) was inoculated with biofilm forming Pseudomonas aeruginosa and treated with ciprofloxacin eye drops, Meanwhile, the first eye of group (3) was inoculated with non- biofilm forming Pseudomonas aeruginosa and treated with intrastromal ciprofloxacin injection, At last, the first eye of the group (4) was inoculated with biofilm forming Pseudomonas aeruginosa and treated with ciprofloxacin eye drops, the second eye was always the control eye.* ***Results:*** *Using ANOVA and LSD tests to compare the means of ulcer diameters in different groups, we found that biofiom is a virulence factor that plays an important role keratitis and there is a great reduction in ulcer size in group receiving intrastromal antibiotic injection compared with the those received eye drops.* ***Conclusion:*** *Biofilm is an important virulence factor that have to be investigated during the diagnostic course of keratitis and intrastromal ciprofloxacin injection is an effective mean of treating keratitis that can provide an alternative way for surgery to treat refractory keratitis.*

INTRODUCTION

Pseudmonas aeruginosa is ubiquitous and opportunistic pathogen that cause many clinical infections. Due to its ability to thrive on most surfaces, it is often found in hospitals and is responsible for many nosocomial infections, respiratory tract infection, urinary tract infection, burns, wound, and eye. It is a major cause of bacterial keratitis. *Pseudomonas* keratitis is a serious disease that can cause scarring and visual disability if not well treated[1,2]. Biofilm formation enables *Pseudomonas* to adhere to and propagate on medical devices like catheters, contact lenses, rough surfaces, etc. [3]

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Keratitis caused by *Pseudomonas* is usually rapidly progressive, characterized by central corneal ulcers and ring abscesses in contrast to gram positive bacteria that usually cause localized abscesses. Histopathological examination shows extensive liquifactive necrosis in the center of the cornea surrounded by PMNs. Ring abscesses contain massive numbers of PMNs[4,5,6]. *Pseudomonas aeruginosa* is an opportunistic bacteria and is able to produce keratitis due to interaction between several hosts and bacterial factors. Enhanced expression of metalloproteinase by keratocytes, secretion of inflammatory cytokines (Interleukin- 1B and TNF-a), in addition to ocular trauma, eye surface diseases and contact lenses are important host factors that predispose to *Pseudomonas aeruginosa* keratitis[7]. Bacterial virulence factors include: several destructive enzymes, especially pseudomonal proteases, exotoxin A, and pseudomonal elastase, lipopolysaccharide and biofilm formation [8,9].

Biofilm production is an important determinant of pathogencity in *Pseudomonas aeruginosa* infections [10], a main cause of persistent ocular disease and antibiotic resistance [1]. Biofilm help the bacteria to adhere to different surfaces and from a complex structure in which bacterial colonies form mushroom like structure with intervening water channels. Bacteria in a biofilm are highly resistant to antibiotics due to several mechanisms: Antibiotic fail to penetrate the biofilm structure, bacteria secrete enzymes that destruct antibiotics or develop efflux pump that pumps antibiotic out. As a result, treatment of infections caused by biofilm-forming bacteria requires higher concentrations of antibiotics than usual [11, 12].

Intrastromal fungal infection has been successfully used to treat fungal keratitis to deliver higher concentrations of antifungal drugs due to poor penetration of antifungal drugs to the stroma, although intrastromal antibiotic injection is not common because antibiotic penetration is better than antifungal [13, 14]. Biofilm is a special condition that needs delivery of much higher antibiotic concentration than usual. Intrastromal injection of antibacterial agents is capable of transmitting higher antibiotic concentration to the stroma [15]. The aim of this work is to suggest an intrastromal injection of antibacterial agents as a promising treatment for refractory keratitis caused by with *Pseudomonas aeruginosa* capable to *in vitro* form biofilm.

METHODOLOGY

Bacterial strains used in the study

A- Isolation of Pseudomonas strains

We collected 105 swabs from patients suffering from corneal ulcers and attending Zagazig University hospitals. We identified 53 isolates, of which as *Pseudomonas aeruginosa*. We identify *Pseudomonas aeruginosa* by producing B-hemolysis on blood agar, green-blue pigment on nutrient agar and a positive oxidase reaction [16]. We used Reference strain *P. aeruginosa (*ATCC 27853) in each run.

B- Testing the isolated Pseudomonas strains for biofilm forming ability.

- **Congo red agar (CRA) method (qualitative detection of biofilm)**

CRA agar media is composed of BHI (37 g/L), sucrose (50 g/L), agar No.1 (10 g/L) and Congo red stain (0.8 g/L) (Oxoid, USA). Congo red was prepared separately in a concentrated aqueous solution and autoclaved at 121°C for 15 min. We incubated Plates aerobically for 24–48 h at 37°C [17]. Biofilm forming colonies appear blackish against the red background media while the non-biofilm forming gives red colonies. Of the 53 isolates obtained 24 isolates showed the ability to form biofilm while 29 isolates were non-biofilm forming as shown in table 1.

- **Tube method (qualitative method)** as described by Abidi et al. [2]. Biofilm forming colonies adhere to tube forming a membrane stained with crystal violet. Of the 53 isolates obtained 28 isolates showed the ability to form biofilm while 25 isolates were non-biofilm forming.
- **Microtitre plate method (quantitative method)** [2]. We classified isolates according to biofilm production, according to the following criteria: non-biofilm producers less than 0.125, weak biofilm producer between 0.125- 0.25 and strong biofilm producers more than 0.25 [18]. Of the 53 isolates obtained, 21 isolates were non biofilm forming (OD <0.1), while 32 isolate formed biofilm on the microtitre plate. 23 were strong biofilm forming (OD> 0.6) and 9 were weak biofilm forming (0.125 > OD< 0.25) (Table 1)

C- Bacterial Isolates used in the study.

Of the 53 *Pseudomonas aeruginosa* isolates, we used forty isolates, twenty non-biofilm forming (as detected by all methods) and twenty strong -biofilm formers all with OD> 0.6.

We performed MIC for ciprofloxacin according to CLSI and EUCAST guidelines. We considered MIC of < 0.5 mg/ul sensitive according to CLSI and EUCAST breakpoints. Of the 53 isolates, 24 (non- biofilm forming) MIC below 0.125mg/L, of the 7 weak biofilm forming isolates MIC ranges from 0.25 to 0.06. 20. Strong biofilm forming isolates had MIC between 0.25 to 0.125. Two isolates which are strong biofilm forming were resistant to ciprofloxacin with MIC 2 mg/ul and > 8 mg/ul. We excluded these resistant strains from the study (Table 1).

Table 1: Biofilm forming ability and MIC for bacterial isolates used in the study

	CRA	***Tube method***	***Mictotitre method***		***MIC Range***
			Number	OD (Range)	
Biofim forming	24	28	23	>0.6	0.25-0.125
			9	0.15>OD>0.25	0.25-0.06
Non-biofilm forming	29	25	21	>0.1	<0.125

D- Inoculum preparation

We incubated each isolate overnight at 37^{0}c on Meuller-Hinton medium, then washed twice by centrifugation at 3000xg in saline (0.9% NaCl in distilled water) for 10 min. We then used 0.5 Mc Farland standards to get 10^{5} cfu/ml [19].

Animals used in the study

In this prospective in vivo experimental study, 50 New Zealand white female rabbits weighing between 1,800 and 2,000 grams were included in the study. The study followed the patterns recommended by Zagazig University research center committee regarding animal use in ophthalmology research.

Each animal received 8.8 mg/kg xylazine and 50mg/kg ketamine IM, and topical proparacain hydrochloride 0.5% to be anesthetized. We used 30 gauge 5/8 needle with 100um fixed banded edge to produce 2mm central and parallel corneal scratches [20].

A- Classification and treatment of animals

- **Control group of animals**: this group included 10 animals; we performed mechanical scratching of both eyes. These animals received no treatment for their eyes. We used this group to assess the effect of eye microflora.
- **Test groups:** We divided rabbits into four groups made of ten females each. After mechanically scratching the eyes, we installed a single drop of bacterial suspension in both eyes.

For each rabbit, the right eye was the eye test and received antibiotic treatment, while, the left eye was the control eye and received only saline.

Test groups were classified as follows:

i- First group: We inoculated animals with *in vitro* biofilm forming *Pseudomonas* and treated them with an intrastromal antibiotic injection

ii- Second group: We inoculated animals with *in vitro* biofilm forming *Pseudomonas* and treated them with eye drops.

iii- Third group: We inoculated animals with *in vitro* non- biofilm forming *pseudomonas* and treated them with intrastromal antibiotic injection.

iv- Fourth group: We inoculated animals with *in vitro* non-biofilm forming *Pseudomonas* and treated them with eye drops.

B- Antibiotic regimen:

Test groups: Inflammation started to appear 12-24 hours after corneal scratching; therapy started 24 hours later (48 hours from the scratching)

Regarding test eyes, groups 2 and 4 received ciprofloxacin eye drops (Ciloxan 0.3% w/v eye drops) hourly for the first two days and then 5 times a day for the remaining 13 days

We injected test eyes of groups 1 and 3 with Ciprofloxacin injection using tuberculin sterile syringes. We used a concentration of 0.3 mg/ml in a single 0.3 ml dose and injected it in five different areas surrounding the ulcer.

C- Measuring the corneal ulcer:

In order to have a contrast, defining the affected area, we applied fluorescein sodium stain to the cornea and then examined the cornea with a cobalt filter of Keeler PLS one portable slit lamp. We recorded the results daily in millimeters in vertical and horizontal meridians of the ulcer

Statistical analysis

We used SPSS version 19 (SPSS Inc., Chicago, IL) in Windows 7 for data processing and statistic. Data were expressed as the number and percentage for qualitative variables and mean ±standard deviation for quantitative one. ANOVA (analysis of variance) test: Used for comparison between more than two different groups of quantitative data which were normally distributed (parametric data). (F) Represents the value of ANOVA test. LSD (least significant difference for comparison between groups) used to show the difference between each two groups. For all above-mentioned statistical tests done, the threshold of significance was fixed at 5% level (P-value).

RESULTS

The clinical pictures of scratched eyes were as follows:

For the control group: There were no signs of infection or inflammation, instead, healing of ulcers occured within 48-72 hours.

For the test group eyes: Ulcers started to show signs of inflammation within 12 h of time after installation of bacteria. In the form of ciliary injection (Redness around the limbus) due to injection of conjunctival vessels, edema of the epithelium and white stromal infiltrate at the edge and the base of the ulcer.

Signs of refractory keratitis were: progression of the lesions characterized by increased conjunctival injection, denudation of the corneal epithelium, increased size of the stromal infiltrate, and peripheral corneal vascularization with no response to treatment for 14 days.

Follow up of rabbits in different groups showed:

Control group: No signs of inflammation appear and complete healing of the scratches occurred within 48-27 hours.

In test groups;

First group: improvement of nine cases after seven days, while one case showed no improvement (perforation was due on the 15^{th} days)

Second group: All cases showed refractory keratitis.

Third group: improvement of all cases within five days

Fourth group: Improvement of eight cases within seven days and two cases showed refractory kertaitis.

Control eyes infected with non-biofilm forming *Pseudomonas aeruginosa* showed better and more rapid healing than those infected with biofilm forming *Pseudomonas aeruginosa* when we compared the mean and SD of corneal defects for the control eyes in all groups. Using the ANOVA test, statistically significant difference appears on the 3rd day P value=0. 49 and then increased, onward to be <0.001 on the 5th day (Table 2, Table 3 and graph 1).

Table 2: The mean and SD of corneal defects for the control and test eyes in all test groups.

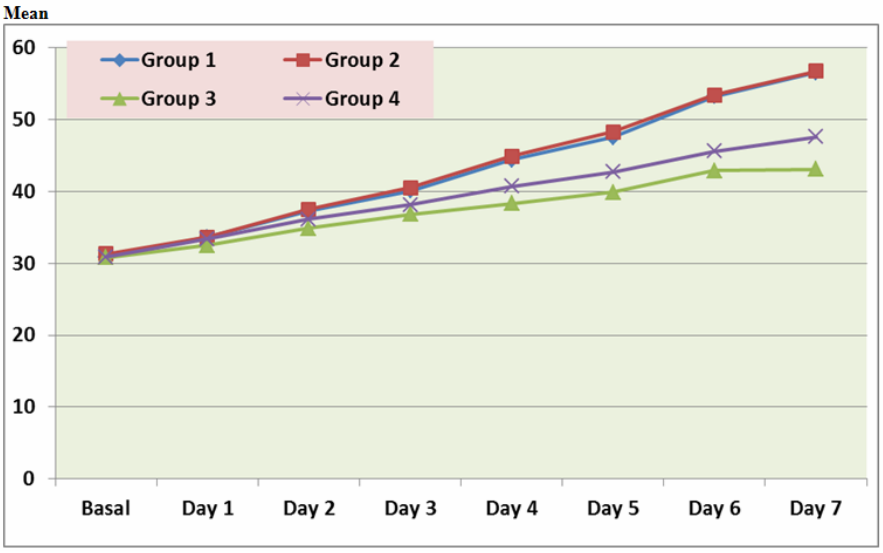
Control and cases groups (Mean/SD)																
	Basal		**Day 1**		**Day 2**		**Day 3**		**Day 4**		**Day 5**		**Day 6**		**Day 7**	
	Control	**Cases**	**Control**	**Cases**	**Control**	**Cases**	**Control**	**Cases**	**Control**	**Cases**	**Control**	**Cases**	**Control**	**Cases**	**Control**	**Cases**
Group 1	30.9± 1.4	30.3± 8.8	33.6± 1.7	33.2± 1.4	37.3± 1.6	28.2± 3	40.1± 2.5	24.1± 5.2	44.4± 2.1	20.5± 8.5	47.5± 2.4	17.4± 9.6	53.2± 3.5	13.8± 11.5	56.5± 3.6	11.9± 12
Group 2	31.3± 1.1	30.6± 1	33.6± 1.7	33.4± 1.6	37.5± 1.6	36.4± 2.6	40.5± 2.7	40.2± 2.8	44.9± 1.6	45±3	48.3± 1.7	50±2. 8	53.4± 3.6	54.1± 3.2	56.7± 3.6	58.5± 2.3
Group 3	30.8± 0.7	30.5± 1	32.5± 0.8	32.7± 1.4	34.9± 1.7	27.9± 5.3	36.8± 3.2	22± 9.7	38.3± 3.8	17.9± 14.1	39.9± 3.7	15.3± 18.6	42.9± 5.2	3.8± 1.2	43.1± 4.8	0
Group 4	30.9± 0.9	30.2± 1	33.4± 1.7	32.5± 1.4	36.1± 3	31.3± 4.9	38.1± 4.6	29.4± 8.2	40.7± 6.4	27.4± 13.2	42.7± 6.8	24.5± 16.4	45.6± 7.8	21.7± 21	47.6± 7.4	20.2± 24.8
F ***	0.375	0.016	1.243	0.715	3.64	10.041	2.86	14.784	7.036	14.571	10.14	15.573	11.06	26.994	19.41	26.165
P. Value	0.771	0.997	0.307	0.549	0.021*	<0.001 **	0.049*	<0.001 **	0.001*	<0.001 **	<0.001 **	<0.00 1**	<0.001 **	<0.00 1**	<0.001 **	<0.001 **

* Significant value
** Highly significant value
*** F: Value of ANOVA test

Table (3): The difference between each two groups of control eyes of the test groups, at different days.

	Group 1	**Group 2**	**Group 3**
Day 2			
Group 2	0.837		0.006*
Group 3	0.01*	0.006 *	
Group 4	0.185	0.127	0.185
Day 3			
Group 2	0.801		0.015*
Group 3	0.028*	0.015*	
Group 4	0.171	0.107	0.38
Day 4			
Group 2	0.746		<0.001**
Group 3	0.001*	<0.001 **	
Group 4	0.036*	0.017 *	0.15
Day 5			
Group 2	0.645		<0.001 **
Group 3	<0.001 **	<0.001 **	
Group 4	<0.011*	0.003 *	0.117
Day 6			
Group 2	0.936		<0.001**
Group 3	<0.001**	<0.001**	
Group 4	0.002 *	0.001*	0.235
Day 7			
Group 2	0.934		<0.001**
Group 3	<0.001**	<0.001**	
Group 4	<0.001**	<0.001**	0.043*

* Significant value
** Highly significant value



Graph 1: Shows that there was a steady increase in the size of the corneal defects in control eyes of test groups in all groups. Both groups one and two shows nearly identical path while groups 3 and 4 shows near results with characteristically less value than the first two groups.

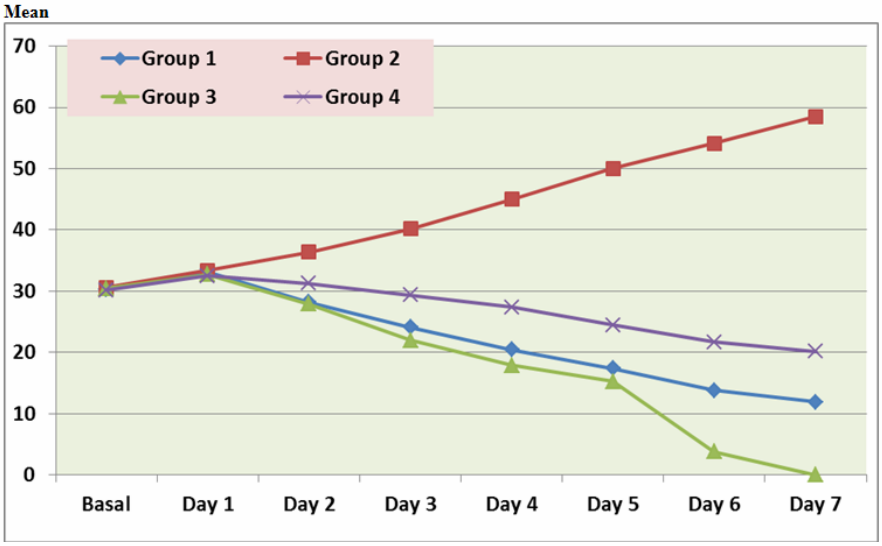
When we compared the effect of using different ways of antibiotic administration for treating different groups, we found that intrastromal antibiotic injection is very effective in improving keratitis caused by *in vitro* biofilm forming *Pseudomonas aeruginosa* compared with conventional eye drops (P value <0.001).

When we examined the difference between each two groups on different days; Intrastromal antibiotic injections significantly help eye to cure rapidly as observed when comparing group1 with group 2 (P value <0.001). When comparing group 3 with group 4, P value was statistically significant in days 2, 3 and 4. While on Day 5 there was no statistically significant difference between two groups as the majority of eyes in group 4 start to heal. The statistically significant difference appears again on day 6 as some cases in group 4 progresses to perforate later (Table 2, Table 4 and graph 2).

Table 4: shows the difference between each two groups at different days.

	Group 1	**Group 2**	**Group 3**
Day 2			
Group 2	<0.001**		<0.001**
Group 3	0.878	<0.001 **	
Group 4	0.058	0.014*	0.042*
Day 3			
Group 2	<0.001 **		<0.001**
Group 3	0.47	<0.001**	
Group 4	0.061	0.002 *	0.012 *
Day 4			
Group 2	<0.001 **		<0.001**
Group 3	0.563	<0.001 **	
Group 4	0.09	0.001	0.026*
Day 5			
Group 2	<0.001 **		<0.001**
Group 3	0.716	<0.001**	
Group 4	0.162	<0.001 **	0.082
Day 6			
Group 2	<0.001 **		<0.001 **
Group 3	0.134	<0.001 **	
Group 4	0.1	<0.001 **	0.006*
Day 7			
Group 2	<0.001 **		
Group 3			
Group 4	0.156	<0.001	

* Significant value ** Highly significant value



Graph 2: **Group 2 shows the worst outcome with a steady increase in the size of the corneal defects. The other three groups, however shows healing with group three being the fastest in healing**

DISCUSSION

Pseudomonas is a virulent organism and is a cause of sever keratitis, which is characterized by rapid and liquifactive necrosis of the cornea[19, 21]. Biofilm formation is a major virulence factor for *Pseudomonas aeruginosa.* Infections associated with biofilm formation are usually difficult to be treated by traditional ways of treatment because biofilm not only provides a protective environment where the bacteria can grow protected from antibacterial but also mediate adhesion of the bacteria to corneal epithelium [17]. Traditional treatment of keratitis caused by *in vitro* biofilm forming *Pseudomonas aeruginosa* using topical administration of ocular antibiotics is thus not sufficient as necrosis of the cornea usually results despite topical antibiotic administration and cause refractory keratitis**.** Intrastromal antibiotic administration provides an effective way for treating these infections and a considerable alternative for surgical intervention [22].

The aim of this study was to evaluate the effect of single intrastromal injection of ciprofloxacin in treating of keratitis caused by *in vitro* biofilm forming *Pseudomonas aeruginosa* and compare it with the conventional use of topical administration of antibiotic.

Ciprofloxacin has been selected for this study because it has an excellent activity against both gram positive and gram negative organisms. Also, it has a dose dependent effect make it a good candidate for single doses of intrastromal injection [23, 24].

Control group of animals was used to investigate the effect of the microflora of rabbit eye, where scratches to the cornea were done under sterile conditions. All eyes of this group show complete healing within 48-72 hours without further intervention. This result can be explained on the base that, these scratches were done under sterile conditions and animals were immune competent, these scratches are usually benign and self limiting [25].

When Comparing the fate of eyes in control eyes of the test group, it was clear that *in vitro* biofilm formation may be an important virulence factor in the development of keratitis that lead to rapid corneal liquefaction and perforation as there were statistically significant difference in the rate of progression of the corneal defect between bacteria that shows difference in *in vitro* biofilm production P value < 0.001. *In vitro* biofilm formation appears to be an important virulence factor because it enables the bacteria to adhere to foreign bodies and rough surface, show more resistance to antibiotic and evade the immune system [26, 27, 28].

Our results illustrated that using conventional eye drops in treating keratitis caused by *in vitro* biofilm forming *Pseudomonas aeruginosa*–even when the organism showed sensitivity to the antibiotic by *in vitro* techniques- is not sufficient and result in refractory keratitis. However, intrastromal injection proved in an effective way to treat such infection. This result can be supported by the fact that intrastromal antifungal injection has proved to be an effective way to treat reluctant cases of fungal keratitis[29,30]. Also, Ling et al and Dryra et al supported the use of intrastraomal antibiotic injection [22, 31]. However, as there is only one case out of nine showed no improvement. It must be considered that *in vitro* biofilm forming is not the only virulence factor, although it is usually associated with other factors like protease enzymes and secretory proteins[32,33]**.**, **i**nvestigation of other virulence factors is to be considered. It may be also important to confirm, that within eye bacteria can form biofilm in vivo by

using methods like fluorescent *in situ* hybridization and electron microscopy [34, 35].

Regarding *Pseudomonas aeruginosa* that did not show the ability to form biofilm in-vitro, our results show that intrastromal antibiotic injection is more efficient that eye drops in treating keratitis, as healing occur within 5 days in 100% of cases. However, in group 4 80% of cases show improvement while treated with eye drops, this considerable healing rate has to be weighed against the fact that intrastromal antibiotic injection is not a complication free maneuver[22, 29]. and thus proper selection of cases indicated for intrastromal antibiotic injections is a must.

Our results show that biofilm formation may be an important virulence factor and a single injection of intrastromal antibacterial antibiotic is significantly efficient in treating *in vitro* biofilm forming *Pseudomonas* keratitis and thus can represent a convenient alternative of surgical intervention [31].

Two cases of group 4 did not show improvement when treated with eye drops, this can be explained on the basis that other virulence factor than biofilm may play an important role in the organism's virulence and thus affect the fate of infection [32, 33].

Finally, we recommend that testing the ability of microorganism to form biofilm is important during the diagnostic procedures of keratitis and whenever ability of biofilm forming is established, intrastromal antibiotic injection is the recommended way for treatment using the sensitive antibiotic

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