総説、著書

著者氏名	論文タイトル名	書籍全体の編集者名	発表紙名、書籍名	出版社名	出版地	出版年	ページ
山田 昌和	加齢と眼表面疾患	-	眼科50		東京都	2008	426-434
山田 昌和	眼科領域の臨床疫学、 効用研究	-	医療62		東京都	2008	695-700
山田 昌和	角膜内皮機能不全の治療	-	医療62		東京都	2008	451-457
山上 聡, 新家 眞, 天野 史郎, 臼井 智彦, 三村 達哉, 横尾 誠一, 青山 佳世, 大沢 稔也, 上羽 悟史, 松島 綱治, 林 孝彦, 田中 香純, 水木 信久, 海老原 信行, 村上 晶, 諸星 計, 宮崎 大, 井上 幸次, Hamrap P, Liu Y, Dana MR, 中野 英樹, 羽藤 晋, 山田 昌和, 羽室 淳爾, 山田 潤, 後藤 晋, 小杉 正明, 鈴木 洋, 北川 全	目の感染と免疫 拒絶反応のない理想的な角膜移植を目指して-全層角膜移植から内皮細胞移植へ-	-	日眼会誌112		東京都	2008	266-278
山田 昌和	先天性遺伝性角膜内皮ジストロフィ	-	新しい眼科26 (2)		東京都	2009	163-166
山田 昌和	角膜・結膜・前眼部の加齢性変化	樋口哲夫/江口秀一郎	眼科プラクティス 22 抗加齢眼科学	文光堂	東京都	2008	23-27
山田 昌和	看護のための最新医学講座	水流 忠彦 編	第2版20巻 眼科疾患	中山書店	東京都	2008	242-249
山田 昌和	感染性角膜炎にステロイドは禁忌だろうか	樋口哲夫/江口秀一郎	眼科診療のコツと落とし穴 4 薬物療法	中山書店	東京都	2008	24-25
山田 昌和	ドライアイの診断にはgolden standardがない	樋口哲夫/江口秀一郎	眼科診療のコツと落とし穴 3 検査・診断	中山書店	東京都	2008	26-27

著者氏名	論文タイトル名	書籍全体の編集者名	発表紙名、書籍名	出版社名	出版地	出版年	ページ
山田　昌和	ドライアイ(乾性角膜炎・Sjogren 症候群)	根木　昭編	眼科プラクティス 23 眼科薬物療法AtoZ	文光堂	東京都	2008	47-48
山田　昌和	びまん性表層角膜炎・点状表層角膜症	根木　昭編	眼科プラクティス 23 眼科薬物療法AtoZ	文光堂	東京都	2008	73－75
若倉　雅登，清澤源弘，山田　昌和.	続・解決、目と視覚の不定愁訴・不明愁訴	（編著）		金原出版	東京都	2008	
山田　昌和	デルモイド	後藤　浩	眼科プラクティス 24 見た目が大事！眼腫瘍	文光堂	東京都	2008	60-61
山田　昌和	デルモリポーマ	後藤　浩	眼科プラクティス 24 見た目が大事！眼腫瘍	文光堂	東京都	2008	62-63
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山田　昌和	保存角膜を利用した輪部デルモイドの治療	後藤　浩	眼科プラクティス 24 見た目が大事！眼腫瘍	文光堂	東京都	2008	91
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山田　昌和	Propionionibacterim acnes 角膜炎	大鹿　哲郎編	眼科プラクティス 18 前眼部アトラス	文光堂	東京都	2007	184

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山田　昌和	デレン	大鹿　哲郎編	眼科プラクティス 18 前眼部アトラス	文光堂	東京都	2007	216
山田　昌和	角膜脂肪変性	大鹿　哲郎編	眼科プラクティス 18 前眼部アトラス	文光堂	東京都	2007	249-250
山田　昌和	細隙灯顕微鏡検査	桶田哲夫編	眼科プラクティス 20 小児科診療	文光堂	東京都	2008	62-64
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山田　昌和	感染性角膜炎.	桶田　哲夫編	眼科プラクティス 20 小児科診療	文光堂	東京都	2008	122-125
山田　昌和	アレルギー性性角膜炎.	桶田　哲夫編	眼科プラクティス 20 小児科診療	文光堂	東京都	2008	126-129
秋山　邦彦，山田　昌和	白内障の診断		白内障	金原出版	東京都	2006	11-16
若倉　雅登，清澤　源弘，山田　昌和.	解決、目と視覚の不定愁訴・不明愁訴	（編著）		金原出版	東京都	2006	

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
山田　昌和、羽藤晋.	角膜移植後の感染症.	前田　直之,黒坂大次郎編	感染症ケース別まるごとマスター	メディカルビュー社	東京都	2006	82-86
後藤　浩,山田昌和,吉川　啓司、飯野　倫子.	整容的に気になる瞼裂斑,母斑,嚢腫への対応	（編著）	眼科開業医のための疑問難問解決策.	診断と治療社	東京都	2006	102-103
山田　昌和	前眼部疾患と両眼視	大月　洋編	両眼視力	金原出版	東京都	2007	115-118

Ⅲ. 研究成果の刊行物・別刷

Fluorophotometric measurement of the precorneal residence time of topically applied hyaluronic acid

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ABSTRACT

Purpose: This study was performed to separately assess the aqueous flow applied with hyaluronic acid, and the behaviour of hyaluronic acid itself on the ocular surface.

Methods: Two different fluorescent dyes, fluorescein sodium dissolved in 0.1% hyaluronic acid (HA) solution and 0.1% fluorescein conjugated with hyaluronic acid (F-HA) dissolved in saline, were used. A volume of 20 µl of tested solution was applied to the eye of 10 healthy volunteers. Fluorescein sodium dissolved in saline served as a control. The fluorescent intensity of the precorneal tear film was measured at the central cornea every minute for 10 min. The turnover rate was calculated using the equation that plots fluorescent intensity against time in a semilog plot and expressed as %/min.

Results: Turnover rates of topically applied 0.1% F-HA, 0.1% HA and saline were 8.1 (SD 3.6)%/min, 21.6 (2.8)%/min, and 31.0 (3.7)%/min, respectively. The turnover rate of F-HA was significantly lower than those of HA and saline ($p = 0.00012$ and $p = 0.00000022$, respectively; Mann–Whitney test). The turnover rate of HA was significantly lower than that of saline ($p = 0.00001$; Mann–Whitney test).

Conclusion: Our results indicate that the bulk aqueous flow applied with HA and the turnover of HA itself are different. HA molecules may adhere to the ocular surface by surface-chemical and/or biochemical properties. The long retention time of HA on the ocular surface may explain the mechanism in which hyaluronic acid has been shown to enhance tear film stability for a few hours.

Dry eye is a common condition, affecting approximately 10–20% of the adult population.[1] The clinical consequences of dry eye may include symptomatic irritation, superficial punctuate keratopathy, corneal erosions and possibly visual acuity problems.[2] Dry eye is considered to be a disorder of the tear film due to tear deficiency or excessive evaporation, which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort.[3]

A variety of treatment modalities have been used for the treatment of dry eye. The majority of these fall into the category of tear substitutes or replacements.[4] Artificial tears, the most frequently used modality for the treatment of dry eye, may be effective in relieving symptoms in mild dry eye by replenishing deficient tear volume. However, in moderate and severe cases of dry eye, artificial tears alone are not enough to relieve the symptoms nor to improve superficial punctuate keratopathy.[4]

Since preliminary reports in the early 1980s,[5–7] several studies have reported that hyaluronic acid is able to improve the symptoms, signs and ocular surface damage associated with dry eye syndrome.[8–11] Hyaluronic acid is a glycosaminoglycan with a viscoelastic rheology. Its relatively high viscosity is believed to improve tear-film stability and to reduce washout from the ocular surface.[12] Hyaluronic acid enhances water retention on the corneal surface, and probably increases corneal wettability.[13] In addition, hyaluronic acid promotes migration of corneal epithelial cells and accelerates the healing of corneal epithelial defects.[14–16] Hyaluronic acid has thus become an important treatment modality for dry eye.

Conflicting results, however, have been obtained regarding the duration of the action of hyaluronic acid on the ocular surface. The residence time of topically applied hyaluronic acid assessed by the tear meniscus height and water evaporation rate from the ocular surface was less than 10 min, although this was significantly longer than that of phosphate-buffered saline.[17 18]

Using an assessment of the tear-film breakup time, however, hyaluronic acid has been shown to enhance tear-film stability for more than a few hours.[19–21] These observations suggest that hyaluronic acid remains on the ocular surface independent of the bulk aqueous flow.

In order to test this hypothesis, we used two different fluorescent dyes, fluorescein sodium dissolved in hyaluronic acid solution and fluorescein conjugated with hyaluronic acid dissolved in saline, in the current study. The former is a well-established dye used to assess the bulk aqueous flow, and the latter dye is used as a tracer to determine the behaviour of hyaluronic acid on the ocular surface. Although the residence time of topically applied hyaluronic acid has been investigated using $^{99}Tc^{m}$ as a tracer,[22–24] we believe that this study is the first report to measure the residence time using a tracer that is associated with hyaluronic acid on the ocular surface.

SUBJECTS AND METHODS

Fluorescent dye and fluorophotometer

Fluorescein hyaluronic acid (F-HA, Mw; 800 000 Da) was purchased from Sigma-Aldrich (St. Louis, MO). F-HA, fluorescein conjugated with hyaluronic acid, was dissolved in a phosphate-buffered saline as 0.1% solution and used as a tracer of hyaluronic acid. Fluorescein sodium (0.001%; Sigma-Aldrich) and 0.1% hyaluronic acid (Mw; 800 000 Da, Sigma-Aldrich) dissolved in a phosphate-buffered saline was used as a tracer of the bulk aqueous flow in the presence of hayaluronic acid. Fluorescein sodium (0.001%) in a phosphate-buffered saline was used as a tracer of the bulk aqueous flow.

A commercial slit-lamp fluorophotometer (Anterior Fluorometer FL-500, Kowa Co., Tokyo) was used. The illuminating light was focused as a 2-mm diameter circle on the surface of the cornea. The emitted light passed through a band-interference filter centred on 565 nm (half bandwidth 25 nm) and was directed to a photomultiplier tube with the band-interference filter centred on wavelengths 490 nm (half bandwidth 30 nm).

F-HA solution (0.5%) was diluted in a phosphate-buffered saline to produce sets of standards ranging from 0.001% to 0.5% in concentration for the calibration. A cuvette was constructed by gluing together two microscope slides and two cover glasses. The cover glasses were sandwiched by two microscope slides in order to provide space for the fluid layer to be 12–15 μm thick. A fresh one was made for each solution. Ten microlitres of calibrating fluids, containing 0.001–0.5% F-HA, was placed into a cuvette. The fluorescent intensity was measured by a slit-lamp fluorophotometer. The interaction of F-HA with the proteins was also tested using a phosphate-buffered saline containing 1% fetal bovine serum.

Measurement of residence time

Ten healthy volunteers (five male and five female) aged 27–44 years (33.8 (SD 6.8) years old, mean (SD)), who had no history of eye disease, except for refractive errors, were enrolled in this study. The principles of the World Medical Association Declaration of Helsinki were followed. The subjects received a full explanation of the procedures, and provided their informed consent for participation prior to the experiment. The protocol was approved by our institutional review board, and all subjects provided their written informed consent.

In our experiments, the subjects were seated in front of the fluorophotometer. The instrument was focused on the central cornea, and the background fluorescent intensity was measured. A volume of 20 μl of tested solution was applied to the eye with an Eppendorf micropipette without making contact. The subjects were then instructed to blink several times to ensure the mixing of the dye. The fluorescent intensity of the precorneal tear film was measured at the central cornea every minute for 10 min. Repeated measurements on different days were made in some subjects to evaluate the repeatability of the test.

The turnover rate is given by the following equation, which plots fluorescent intensity against time in a semilog plot:

$$F = F_0 \exp(-kt)$$

where F is the fluorescent intensity at time (t); F_0 is the fluorescent intensity at time zero; k is the turnover rate; and t is the time in minutes.[25] The turnover rate was calculated using the equation and expressed as %/min. The regression fit of the log of the fluorescent intensity was recorded as the regression coefficient.

In all cases of 0.1% F-HA and 0.1% hyaluronic acid, this regression became a straight line. In cases of saline, however, this regression sometimes showed a biphasic response: an initial faster and a subsequent lower turnover rate. When the turnover rate of saline became biphasic, the subsequent lower turnover rate was used as the flow rate of saline.[25]

All results are presented as the mean ± 1 standard deviation (SD). Statistical significance was calculated by comparing results using the Mann–Whitney test. A value of $p<0.05$ was considered to indicate statistical significance.

RESULTS

Calibration of F-HA

The calibration of the fluorescent intensities against the concentrations of 0.001–0.5% F-HA is shown in fig 1. The relationship between the fluorescent intensities and the concentrations of F-HA was linear ($r^2 = 0.995$). The data generated by this method were consistent and reproducible. The fluorescent intensities of F-HA were unaffected by the presence of 1% fetal bovine serum (data not shown).

Turnover-rate measurements

A typical result of turnover-rate measurements obtained from one subject is shown in fig 2. In the presented case, the fluorescent intensities of 0.1% F-HA decayed with time at a flow rate of 7.6%/min, which was lower than those of fluorescein sodium in 0.1% hyaluronic acid (19.4%/min) and in saline (28.1%/min).

The turnover rates of topically applied F-HA, hyaluronic acid and saline obtained from 10 subjects were 8.1 (3.6)%/min, 21.6 (2.8)%/min, and 31.0 (3.7)%/min, respectively (table 1). The turnover rate of F-HA was significantly lower than those of hyaluronic acid and saline ($p = 0.00012$, and $p = 0.00000022$, respectively; Mann–Whitney test). The turnover rate of F-HA was significantly lower than that of saline ($p = 0.00001$; Mann–Whitney test).

DISCUSSION

In the current study, we used two different fluorescent dyes, fluorescein sodium dissolved in hyaluronic acid solution and F-HA solution, to separately assess the aqueous flow applied with hyaluronic acid, and the behaviour of hyaluronic acid itself on the ocular surface. Our results indicate that there are two different aspects of the duration of topically applied hyaluronic acid.

Hyaluronic acid has a high-molecular-weight, naturally occurring glycosaminoglycan. Its relatively high viscosity is believed to reduce washout from the ocular surface.[13] The residence time of topically applied hyaluronic acid has previously been investigated by using gamma scintigraphic methods.[22–24] Snibson and associates[22 24] reported that hyaluronic acid had prolonged ocular residence times in comparison

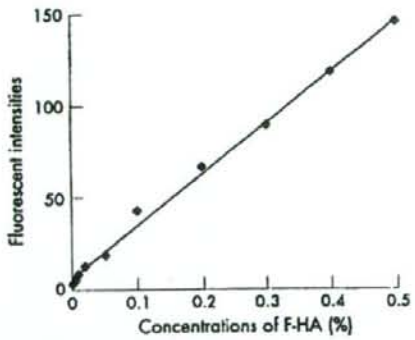

Figure 1 Calibration of the fluorescent intensities against the concentrations of flurorescein hyaluronic acid (F-HA). The relationship between the fluorescent intensities and the concentrations of F-HA was linear ($r^2 = 0.995$).

Table 1 Turnover rates of topically applied 0.1% fluorescein hyaluronic acid (F-HA), 0.1% hyaluronic acid and saline obtained from 10 subjects

Subject no.	Turnover rate (%/min)		
	F-HA	Hyaluronic acid	Saline
1	7.6	19.4	28.1
2	12.2	22.6	31.0
3	2.9	22.6	31.8
4	9.5	20.4	33.2
5	4.6	21.4	33.4
6	8.6	22.2	37.1
7	15.2	24.2	29.6
8	8.4	20.4	24.1
9	5.3	17.1	27.8
10	7.1	21.3	33.9
Mean (SD)	8.1 (3.6)	21.7 (2.8)	31.0 (3.7)

The turnover rate of F-HA was significantly lower than those of hyaluronic acid and saline ($p = 0.00012$ and $p = 0.00000022$, respectively; Mann–Whitney test).

with a buffered saline solution, a solution containing polyvinyl alcohol or hydroxypropylmethylcellulose. In the current study, the turnover rate of the 0.1% hyaluronic acid solution (21.6 (2.8)%/min) was significantly lower than that of the saline (31.0 (3.7)%/min). Our result is considered to be in good accordance with the previous studies using scintigraphic methods.[22–24] This effect, however, appears to be transient, because 90% of the hyaluronic acid solution was calculated to be cleared from the ocular surface 10.7 min after instillation. This result is also consistent with the duration of topically applied hyaluronic acid assessed by the tear meniscus height and water evaporation rate from the ocular surface.[17 18]

The most interesting finding of the current study is that the turnover rate of F-HA (8.1 (3.6)%/min) was approximately one-third of the 0.1% hyaluronic acid solution (21.6 (2.8)%/min). This result indicates that the bulk aqueous flow applied with hyaluronic acid and the turnover of hyaluronic acid itself on the ocular surface are different. Besides viscosity, hyaluronic acid molecules may adhere to the ocular surface by surface-chemical and/or biochemical properties, because hyaluronic acid is known to bind with fibronectin and CD44, a cell surface adhesion molecule which has been found on corneal epithelial cells.[16 26] Snibson and associates[24] made a similar consideration based on their scintigraphic results. They, however, also mentioned the limitation of their methodology and the necessity of tracers that directly associate with hyaluronic acid. We believe that F-HA is a useful tracer to determine the behaviour of topically applied hyaluronic acid. According to our results, the times for 90% and 99% of hyaluronic acid to be cleared from the ocular surface were calculated to be 28.8 min and 57.6 min after instillation, respectively. The long retention time of hyaluronic acid in the ocular surface may explain the fact that hyaluronic acid has been shown to enhance tear-film stability for more than a few hours.[19–21]

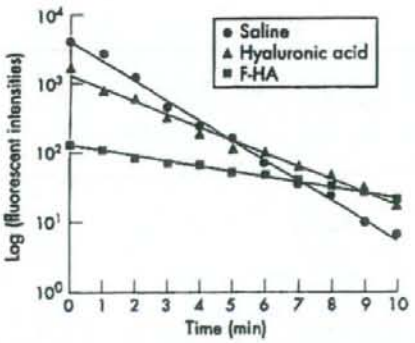

Figure 2 Typical result of the turnover rate measurements obtained from one subject. In the presented case, the fluorescent intensities of 0.1% fluorescein hyaluronic acid (F-HA) decayed with time at a flow rate of 7.6%/min, which was lower than those of fluorescein sodium in 0.1% hyaluronic acid (19.4%/min) and in saline (28.1%/min).

Besides its biological effects on the corneal epithelial cells, hyaluronic acid appears to have two beneficial effects for the treatment of dry eye syndrome. First, it reduces the bulk aqueous flow by its viscosity and increases tear volume for a limited time, as do other viscous agents, such as chondroitin sulfate, polyvinyl alcohol and hydroxypropylmethylcellulose.[22] Second, hyaluronic acid remains on the ocular surface for a longer time, in order to increase corneal wettability and to retain tear fluid on the corneal surface.[12 13] This effect may be unique for hyaluronic acid, although it should be confirmed by further investigations.

Competing interests: The authors have no proprietary interest in any materials in this manuscript.

REFERENCES

1. **Johnson ME,** Murphy PJ. Changes in the tear film and ocular surface from dry eye syndrome. *Prog Retin Eye Res* 2004;**23**:449–74.
2. **Holy FJ,** Lemp MA. Tear physiology and dry eyes. *Surv Ophthalmol* 1977;**22**:69–87.
3. **Lemp MA.** Report of the National Eye Institute/Industry workshop on clinical trials in dry eyes. *CLAO J* 1995;**21**:221–32.
4. **Nelson JD,** Farris RL. Sodium hyaluronate and polyvinyl alcohol artificial tear preparations. *Arch Ophthalmol* 1988;**106**:484–7.
5. **Polack FM,** McNiece MT. The treatment of dry eyes with Na hyaluronate (Healon): a preliminary report. *Cornea* 1982;**1**:133–6.
6. **Stuart JC,** Linn JG. Dilute sodium hyaluronate (Healon) in the treatment of ocular surface disorders. *Ann Ophthalmol* 1985;**17**:190–2.
7. **DeLuise VP,** Peterson WS. The use of topical Healon tears in the management of refractory dry-eye syndrome. *Ann Ophthalmol* 1984;**16**:823–4.
8. **Aragona P,** Papa V, Micali A, *et al.* Long term treatment with sodium hyaluronate-containing artificial tears reduces ocular surface damage in patients with dry eye. *Br J Ophthalmol* 2002;**86**:181–4.
9. **Limberg MB,** McCaa C, Kissling GE, *et al.* Topical application of hyaluronic acid and chondroitin sulfate in the treatment of dry eyes. *Am J Ophthalmol* 1987;**103**:194–7.
10. **Shimmura S,** Ono M, Shinozaki K, *et al.* Sodium hyaluronate eye drops in the treatment of dry eyes. *Br J Ophthalmol* 1995;**70**:1007–11.
11. **Condon PI,** McEwen CG, Wright M, *et al.* Double blind, randomized, placebo controlled, crossover, multicentre study to determine the efficacy of a 0.1% (w/v) sodium hyaluronate solution (Fermavisc) in the treatment of dry eye syndrome. *Br J Ophthalmol* 1999;**83**:1121–4.
12. **Nakamura S,** Okada S, Umeda Y, *et al.* Development of a rabbit model of tear film instability and evaluation of viscosity of artificial tear preparations. *Cornea* 2004;**23**:390–7.
13. **Nakamura M.** Hikida M, Nakano T, *et al.* Characterization of water retentive properties of hyaluronan. *Cornea* 1993;**12**:433–6.
14. **Nishida T,** Nakamura M, Mishima H, *et al.* Hyaluronan stimulates corneal epithelial migration. *Exp Eye Res* 1991;**53**:753–8.
15. **Sugiyama T,** Miyauchi S, Machida A, *et al.* The effect of sodium hyaluronate on the migration of rabbit corneal epithelium. The effect of topical administration. *J Ocular Pharmacol* 1991;**7**:53–64.

16. **Gomes JAP,** Amankwah R, Powell-Richards A, *et al*. Sodium hyaluronate promotes migration of human corneal epithelial cells in vitro. *Br J Ophthalmol* 2004;**88**:821–5.
17. **Watanabe T,** Kawashima M, Kawai M, *et al*. Effect of sodium hyaluronate eyedrops on tear meniscus height determined by fluorescein photography. [in Japanese] *Folia Ophthalmol Jpn* 2004;**55**:369–73.
18. **Tsubota K,** Yamada M. Tear evaporation from the ocular surface. *Invest Ophthalmol Vis Sci* 1992;**33**:2942–50.
19. **Mengher LS,** Pandher KS, Bron AJ, *et al*. The effect of sodium hyaluronate (0.1%) on break-up time (NIBUT) in patients with dry eyes. *Br J Ophthalmol* 1986;**70**:442–7.
20. **Hamano T,** Horimoto K, Lee M, *et al*. Evaluation of the effect of sodium hyaluronate ophthalmic solution on tear film stability. [in Japanese] *J Jpn Ophthalmol Soc* 1993;**97**:928–32.
21. **Johnson ME,** Murphy PJ, Boulton M. Effectiveness of sodium hyaluronate eyedrops in the treatment of dry eye. *Graefe's Arch Clin Exp Ophthalmol* 2006;**244**:109–12.
22. **Snibson GR,** Greaves JL, Soper NDW, *et al*. Precorneal residence times of sodium hyaluronate solutions studied by quantitative gamma scintigraphy. *Eye* 1990;**4**:594–602.
23. **Gurny R,** Ryser JE, Tabatabay C, *et al*. Precorneal residence time in humans of sodium hyaluronate as measured by gamma scintigraphy. *Graefe's Arch Clin Exp Ophthalmol* 1990;**228**:510–2
24. **Snibson GR,** Greaves JL, Soper NDW, *et al*. Ocular surface residence times of artificial tear solutions. *Cornea* 1992;**11**:288–93.
25. **Mishima S,** Gasset A, Klyce Jr SD, *et al*. Determination of tear volume and tear flow. *Invest Ophthalmol Vis Sci* 1966;**5**:264–76.
26. **Asari A,** Morita M, Sekiguchi T, *et al*. Hyaluronan, CD44 and fibronectin in rabbit corneal wound healing. *Jpn J Ophthalmol* 1996;**40**:18–25.

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In Vitro Susceptibilities of Bacterial Isolates From Conjunctival Flora to Gatifloxacin, Levofloxacin, Tosufloxacin, and Moxifloxacin

Masakazu Yamada, M.D., Shin Hatou, M.D., and JunkoYoshida, M.D.

Purpose. To evaluate and compare the in vitro susceptibilities of various fluoroquinolones (i.e., gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin) against conjunctival bacterial flora. **Methods.** Two hundred sixty-six bacterial isolates were collected from the conjunctival sacs of 251 eyes of 224 patients (118 females and 106 males) ranging in age from 6 to 91 years old, who were scheduled for intraocular surgery at National Tokyo Medical Center. The minimum inhibitory concentration (MIC) was determined by broth dilution testing. **Results.** Of 266 isolates, 258 (97.0%) strains were gram-positive bacteria and eight (3.0%) strains were gram-negative bacteria. The MIC_{90} values of gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin against α-hemolytic streptococci were 0.39 μg/mL, 1.56 μg/mL, 0.39 μg/mL, and 0.20 μg/mL, respectively. The MIC_{90} values of gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin against *Staphylococcus aureus* were 1.56 μg/mL, 3.13 μg/mL, 0.78 μg/mL, and 0.78 μg/mL, respectively. The MIC_{90} values of gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin against *Staphylococcus epidermidis* were 1.56 μg/mL, 3.13 μg/mL, 3.13 μg/mL, and 0.78 μg/mL, respectively. **Conclusions.** Although the clinical usefulness and efficacy of newer fluoroquinolones remains to be defined by clinical outcomes, the current study provides data for predicting relative in vivo potency among the fluoroquinolones.

Key Words: Bacterial flora—Conjunctiva—Drug resistance—Fluoroquinolone—Ocular infection.

Fluoroquinolones are the newest family of antibacterial agents used in the treatment of ocular infections.[1–5] In Japan, ofloxacin was the first fluoroquinolone introduced for topical ophthalmic use in 1987. Since then, six other fluoroquinolones, norfloxacin, lomefloxacin, levofloxacin, gatifloxacin, tosufloxacin, and moxifloxacin, have been approved for clinical use as eyedrops in Japan. In addition to these compounds, ciprofloxacin has been used clinically in other countries.

Double-masked, randomized clinical trials have shown that single-agent fluoroquinolone therapy using ofloxacin[4] or ciprofloxacin[5] against bacterial keratitis is comparable in efficacy to combining fortified β-lactum agents and aminoglycosides. Their bactericidal activity against the most common gram-positive and gram-negative ocular pathogens is generally excellent, and their high potency has made fluoroquinolones a common choice for the treatment and prevention of ocular infections.

However, as with other antibiotic agents, continued use in a population raises the issue of emerging resistance.[6] Since the introduction of fluoroquinolones for ophthalmic use, the reported incidence of in vitro resistance to fluoroquinolones among bacteria isolated from patients with bacterial keratitis and endophthalmitis has been steadily increasing.[6,7] A previous study by the authors[8] reviewed the database of bacterial flora cultured preoperatively from the conjunctival sac of 1,455 Japanese patients between 1995 and 1999. The incidence of in vitro resistance of bacterial isolates to ofloxacin increased from 13.5% in 1995 to 32.8% in 1999. Although ofloxacin was changed to levofloxacin in 2000, the incidence of resistance to levofloxacin gradually increased from 14.5% in 2000 to 20.5% in 2002.[9]

Some newer fluoroquinolones have been introduced for topical ophthalmic use: gatifloxacin, tosufloxacin, and moxifloxacin. They are sometimes categorized as third-generation fluoroquinolones (i.e., tosufloxacin) and fourth-generation fluoroquinolones (i.e., gatifloxacin and moxifloxacin).[10] Although the clinical benefits of these newer fluoroquinolones have yet to be fully established, their attributes suggest a potential role for the prevention of the increasing incidence of fluoroquinolone resistance among bacterial ocular pathogens. Gatifloxacin and moxifloxacin, especially, which are called 8-methoxyfluoroquinolones, are less likely to engender resistance from single-step topoisomerase mutations. It requires a double mutation in DNA gyrase and topoisomerase IV to establish resistance to 8-methoxyfluoroquinolones.[10] Other potentially beneficial features of 8-methoxyfluoroquinolones are enhanced gram-positive activity relative to older fluoroquinolones and improved drug delivery into the anterior segment of the eye.

This study compared the in vitro effectiveness of bacterial flora cultured preoperatively from the conjunctival sac of patients undergoing intraocular surgery to gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin.

MATERIALS AND METHODS

Two hundred sixty-six bacterial isolates were collected from the conjunctival sacs of 251 eyes of 224 patients (118 females and 106 males) ranging in age from 6 to 91 years old, who were scheduled

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TABLE 1. *Total Number of 266 Ocular Clinical Isolates*

Bacteria	No. of isolates
All isolates	266 (100%)
Gram-positive species	258 (97.0%)
α-Hemolytic streptococci	43
Staphylococcus aureus	56
Methicillin-sensitive *S. aureus*	48
Methicillin-resistant *S. aureus*	8
Staphylococcus epidermidis	140
Methicillin-sensitive *S. epidermidis*	59
Methicillin-resistant *S. epidermidis*	81
Enterococcus faecalis	14
Others	5
Gram-negative species	8 (3.0%)
Pseudomonas species	6
Others	2

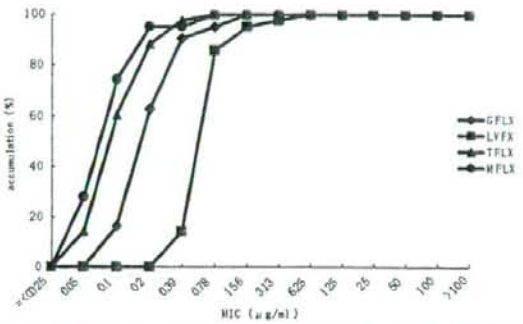

FIG. 1. The accumulation curves of the minimum inhibitory concentration (MIC) of the four fluoroquinolones against α-hemolytic streptococci (n = 43). Circle, moxifloxacin; triangle, tosufloxacin; diamond, gatifloxacin; square, levofloxacin.

for intraocular surgery at National Tokyo Medical Center from December 2004 to November 2005. The principles of the World Medical Association Declaration of Helsinki were followed. Each subject received a thorough explanation of the purpose of the study and all procedures involved in the study and provided written informed consent before enrollment. Approval for this investigation was granted by the Committee for the Protection of Human Subjects of National Tokyo Medical Center.

Scrapes of the inferior conjunctival fornix were taken preoperatively by using a sterile cotton swab without a topical anesthetic. The samples were immediately inoculated into the heart infusion bouillon, incubated for 24 hours at 37°C, and then inoculated into blood agar and MacConkey agar before incubating again for 24 hours at 37°C. Positive cultures were stored at −80°C until broth dilution testing to determine the minimum inhibitory concentration (MIC).

For the broth dilution testing, frozen microdilution MIC plates with gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin were prepared according to the recommendations in the Clinical and Laboratory Standards Institute's Performance Standards for Antimicrobial Susceptibility Testing.[11] In this method, the concentration of antibiotic remains constant in each well, so organisms are exposed to identical concentrations for the duration of the test. Fluoroquinolone concentration ranged from 0.025 to 100 μg/mL.

The MIC of oxacillin (MPIPC) was also determined by the same method for *Staphylococcus aureus* and *Staphylococcus epidermidis*. When the MIC was greater than 4 μg/mL for *S. aureus* and 0.5 μg/mL for *S. epidermidis*, the strain was classified as methicillin-resistant *S. aureus* (MRSA) or methicillin-resistant *S. epidermidis* (MRSE).[11]

RESULTS

Bacterial Isolates From Conjunctival Flora

Table 1 shows the type and frequency of the 266 ocular clinical isolates. Of the total 266 isolates, 258 (97.0%) strains were gram-positive and eight (3.0%) strains were gram-negative. *S. epidermidis* was the most common form of gram-positive bacteria with 140 strains, followed by 56 strains of *S. aureus*, 43 strains of α-hemolytic streptococci, and 14 strains of *Enterococcus faecalis*. Six of the eight strains of gram-negative bacteria were *Pseudomonas* species.

Antibacterial Activity of Fluoroquinolones

Figure 1 shows the MIC accumulation curves for the four study agents against α-hemolytic streptococci. The MIC_{90} values of gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin were 0.39 μg/mL, 1.56 μg/mL, 0.39 μg/mL, and 0.20 μg/mL, respectively (Table 2). All strains of α-hemolytic streptococci were susceptible to all four study agents.

Figure 2 shows the MIC accumulation curves for the four study agents against *S. aureus*. Fifty-six isolates of *S. aureus* contained eight MRSA strains. The MIC_{90} values of gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin were 1.56 μg/mL, 3.13 μg/mL, 0.78 μg/mL, and 0.78 μg/mL, respectively (Table 2). Almost all *S. aureus* strains susceptible to MPIPC were also susceptible to all four study agents, whereas MRSA strains were resistant to all four fluoroquinolones in general.

Figure 3 shows the MIC accumulation curves for the four study agents against *S. epidermidis*. One hundred forty isolates of *S. epidermidis* contained 81 MRSE strains. The accumulation curves of the MICs of all four fluoroquinolones were not linear but

TABLE 2. *The Minimum Inhibitory Concentration of Four Fluoroquinolones Against Bacterial Isolates*

Bacteria	MIC_{90}, μg/mL (range of MIC)			
	Gatifloxacin	Levofloxacin	Tosufloxacin	Moxifloxacin
α-Hemolytic streptococci (n = 43)	0.39 (0.1–1.56)	1.56 (0.39–6.25)	0.39 (0.05–0.78)	0.20 (0.05–0.78)
Staphylococcus aureus (n = 56)	1.56 (0.05–50)	3.13 (0.1–>100)	0.78 (≤0.025–25)	0.78 (≤0.025–50)
Staphylococcus epidermidis (n = 140)	1.56 (0.05–12.5)	3.13 (0.1–25)	3.13 (≤0.025–12.5)	0.78 (≤0.025–12.5)
Enterococcus faecalis (n = 14)[a]	0.39 (0.39–25)	1.56 (0.78–25)	0.2 (0.2–12.5)	0.2 (0.2–12.5)
Pseudomonas species (n = 6)[a]	0.78 (0.39–1.56)	0.78 (0.39–1.56)	0.2 (0.2–0.39)	1.56 (0.78–3.13)

[a]For *Enterococcus faecalis* and *Pseudomonas* species, the medians of the minimum inhibitory concentration (MIC) are listed because of insufficient sample numbers.

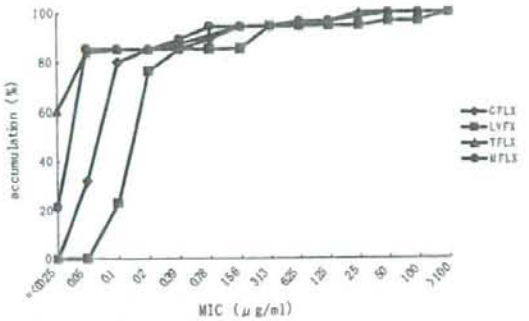

FIG. 2. The accumulation curves of the minimum inhibitory concentration (MIC) of the four fluoroquinolones against *Staphylococcus aureus* (n = 56). Triangle, tosufloxacin; circle, moxifloxacin; diamond, gatifloxacin; square, levofloxacin.

sigmoid and suggested the presence of drug-resistant strains. The MIC_{90} values of gatifloxacin, levofloxacin, tosufloxacin, and moxifloxacin were 1.56 μg/mL, 3.13 μg/mL, 3.13 μg/mL, and 0.78 μg/mL, respectively (Table 2).

The MICs of fluoroquinolones and MPIPC were compared against *S. aureus* and *S. epidermidis*. The MIC of each fluoroquinolone was closely correlated with that of MPIPC for *S. aureus*, suggesting that MRSA was resistant to each fluoroquinolone. In contrast, there was no correlation between the MICs of MPIPC and the fluoroquinolones against *S. epidermidis* (Table 3).

TABLE 3. *Correlation Between the Minimum Inhibitory Concentration of Four Fluoroquinolones and Oxacillin Against* Staphylococcus aureus *and* Staphylococcus epidermidis

Correlation versus oxacillin	Coefficient (P value)			
	Gatifloxacin	Levofloxacin	Tosufloxacin	Moxifloxacin
Staphylococcus aureus (n = 56)	0.85[a]	0.88[a]	0.85[a]	0.84[a]
Staphylococcus epidermidis (n = 140)	0.37	0.11	0.34	0.33

[a]P<0.0001.

DISCUSSION

One possible limitation of the current study is that samples originated from conjunctival cultures obtained from patients scheduled for intraocular surgery. Samples therefore should be regarded as conjunctival flora rather than ocular pathogens, because the patients did not have bacterial diseases at the time of sampling. However, in common ocular infections, such as bacterial conjunctivitis and bacterial keratitis, pathogens are frequently the normal bacterial flora residing on the ocular surface,[12,13] even in cases of postoperative endophthalmitis, which is an infrequent but devastating form of ocular infection.[14] In one study, organisms isolated from the vitreous were genetically identical to those collected from the ocular surface in 68% to 82% of patients with postoperative endopthalmitis,[15] suggesting it is a valid approach to study in vitro susceptibility of bacteria isolated from conjunctival flora to various fluoroquinolones.

A serious concern is a drug resistance. In the current study, fluoroquinolones and MPIPC were compared with respect to their MICs against *S. aureus* and *S. epidermidis*. In *S. aureus*, the MICs of each fluoroquinolone were closely related to those of MPIPC, suggesting that MRSA was also insusceptible to most fluoroquinolones. Therefore, when ocular pathogens are identified as MRSA, the use of fluoroquinolones, even gatifloxacin and moxifloxacin, are not recommended for treatment. In contrast, there was no relationship between the MICs of MPIPC and the fluoroquinolones for *S. epidermidis*. These results indicate that some strains of *S. epidermidis* are not susceptible to fluoroquinolones, regardless of their susceptibility to MPIPC. Differences between *S. aureus* and *S. epidermidis* may be of interest when studying drug resistance mechanisms.

This study also found that some strains of *S. epidermidis* were highly resistant to levofloxacin and tosufloxacin, but not to moxifloxacin and gatifloxacin. These strains were considered to be low-level fluoroquinolone-resistant bacterial isolates. These data may favor the selection of moxifloxacin and gatifloxacin, rather than levofloxacin or tosufloxacin, for prophylactic use or the treatment of ocular infections.

In this study, newer fluoroquinolones showed more potent antibacterial activity than levofloxacin, the fluoroquinolone most commonly used in Japan. The current results support the contention that the fourth-generation fluoroquinolones have enhanced activity against gram-positive bacteria while retaining potency against most gram-negative bacteria. These newer fluoroquinolones have also improved penetration into the ocular tissue.[10] Increased in vivo efficacy of these newer fluoroquinolones in some animal models of ocular infections have also been reported.[16] Some caution, however, should be exercised when interpreting the data presented. The success or failure of a given therapy is not necessarily predicted by MIC_{90} values, because the MIC_{90} determined through in vitro assays may not directly correlate with clinical results. The actual efficacy of these newer fluoroquinolones remains to be defined by clinical outcomes. The data of the current study may be used to predict relative in vivo potency among the fluoroquinolones.

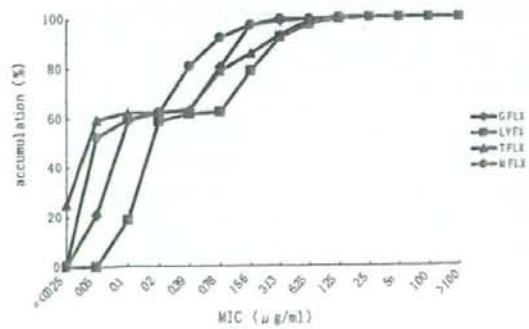

FIG. 3. The accumulation curves of the minimum inhibitory concentration (MIC) of the four fluoroquinolones against *Staphylococcus epidermidis* (n = 140). Triangle, tosufloxacin; circle, moxifloxacin; diamond, gatifloxacin; square, levofloxacin.

REFERENCES

1. O'Brien TP. Bacterial keratitis. In: Krachmer JH, Mannis MJ, Holland EJ, eds. *Cornea and External Disease: Clinical Diagnosis and Management, vol. II.* St. Louis, Mosby-Year Book, 1997, pp 1139–1190.

2. Neu HC. Microbiologic aspects of fluoroquinolones. *Am J Ophthalmol* 1991;112:S15–S24.
3. Leibowitz HM. Clinical evaluation of ciprofloxacin 0.3% ophthalmic solution for treatment of bacterial keratitis. *Am J Ophthalmol* 1991; 112:S34–S47.
4. O'Brien TP, Maquire MG, Fink NE, et al. Efficacy of ofloxacin versus cefazolin and tobramycin in the therapy for bacterial keratitis: Report from the Bacterial Keratitis Study Research Group. *Arch Ophthalmol* 1995;113:1257–1265.
5. Hyndiuk RA, Eiferman RA, Caldwell DR, et al. Comparison of ciprofloxacin ophthalmic solution 0.3% to fortified tobramycin-cefazolin in treating bacterial corneal ulcers. *Ophthalmology* 1996;103: 1854–62.
6. Chalita MG, Hüfling-Lima AN, Paranhos A, et al. Shifting trends in in vitro antibiotic susceptibilities for common ocular isolates during period of 15 years. *Am J Ophthalmol* 2004;137:43–51.
7. Goldstein MH, Kowalski RP, Gordon YJ. Emerging fluoroquinolone resistance in bacterial keratitis. *Ophthalmology* 1999;106:1313–1318.
8. Kurokawa N, Hayashi K, Konishi M, et al. Increasing ofloxacin resistance of bacterial flora from conjunctival sac of preoperative ophthalmic patients in Japan. *Jpn J Ophthalmol* 2002;46:586–589.
9. Sakurai M, Hayashi K, Obazawa M, et al. Levofloxacin-resistant bacteria in conjunctival flora of patients undergoing intraocular surgery [in Japanese]. *J Eye* 2005;22:97–100.
10. Hwang DG. Fluoroquinolone resistance in ophthalmology and the potential role for newer ophthalmic fluoroquinolones. *Surv Ophthalmol* 2004;49:S79–S83.
11. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobacterial Susceptibility Testing: Sixteenth informational supplement.* 2006;25:M100–S16.
12. Graves A, Henry M, O'Brien TP, et al. In vitro susceptibilities of bacterial ocular isolates to fluoroquinolones. *Cornea* 2001;20:301–305.
13. Stern GA. Contact lens associated bacterial keratitis: Past, present and future. *CLAO J* 1998;24:52–56.
14. Liesegang TJ. Use of antimicrobials to prevent postoperative infection in patients with cataract. *Curr Opin Ophthalmol* 2001;12:68–74.
15. Han DP, Wisniewski SR, Wilson LA, et al. Spectrum and susceptibilities of microbiologic isolates in the endophthalmitis vitrectomy study. *Am J Ophthalmol* 1996;122:1–17.
16. Tungsiripat T, Sarayba MA, Kaufman MB, et al. Fluoroquinolone therapy in multiple-drug resistant staphylococcal keratitis after lamellar keratectomy in a rabbit model. *Am J Ophthalmol* 2003; 136:76–81.

Mutations in the quinolone resistance determining region in *Staphylococcus epidermidis* recovered from conjunctiva and their association with susceptibility to various fluoroquinolones

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ABSTRACT

Background: *Staphylococcus epidermidis* is one of the prominent pathogens in ocular infection. The prevalence of mutations in the quinolone resistance determining region (QRDR) area in *S epidermidis* isolated from the ocular surface and its association with fluoroquinolone resistance has not been fully elucidated.

Methods: Mutations in the QRDR of *gyrA*, *gyrB*, *parC*, and *parE* genes of 138 isolates of *S epidermidis* recovered from the human conjunctival flora were analysed. The minimal inhibitory concentrations (MICs) of four fluoroquinolones (levofloxacin, gatifloxacin, moxifloxacin and tosufloxacin) against these isolates were also determined using agar dilution methods.

Results: The MIC_{50} values of levofloxacin, gatifloxacin, moxifloxacin and tosufloxacin were 3.13, 1.56, 0.78 and 3.13 μg/ml, respectively. The MIC values of all fluoroquinolones showed a bimodal distribution (susceptible strain and less susceptible strain). Mutations with amino acid substitution in the QRDR were present in 70 (50.7%) isolates. 19 different combinations of mutations were detected: 3 isolates (2.2%) had four mutations, 8 (5.8%) had three mutations, 43 (31.2%) had double mutations and 16 (11.6%) had single mutations. Isolates with mutations in the QRDR of both *gyrA* and *parC* (n = 53) were less susceptible to fluoroquinolones.

Conclusions: The present findings show that approximately half the *S epidermidis* isolates from the normal human conjunctiva have mutation(s) in the QRDR. The presence of mutations in both *gyrA* and *parC* is strongly associated with reduced susceptibility to fluoroquinolones.

Staphylococcus epidermidis is one of the most prominent causes of conjunctivitis, keratitis and endophthalmitis.[1–4] Although the relative frequency of different organisms as causative agents in keratitis varies during different periods and in different geographical regions, *S epidermidis* is among the most frequently encountered organisms in clinical studies conducted in the USA, Germany and Japan.[3–5] It is the most common bacterial isolate in most large studies of acute postoperative endophthalmitis.[7 8]

The fluoroquinolones are the newest family of antibacterial agents used in the treatment of ocular infections.[2 3 9–11] In Japan, ofloxacin was the first fluoroquinolone introduced for topical ophthalmic use in 1987. Since then, six other fluoroquinolones—norfloxacin, lomefloxacin, levofloxacin (LVFX), gatifloxacin (GFLX), tosufloxacin (TFLX) and moxifloxacin (MFLX)—have been approved for clinical use as eye drops in Japan. In addition to these compounds, ciprofloxacin has been used clinically in other countries. Their bactericidal activity against the most frequently observed Gram-positive and Gram-negative ocular pathogens is generally excellent and their high potency has made them a common choice for the treatment and prevention of ocular infections.

However, as with other antibiotic agents, continued use in a population raises the issue of emerging resistance.[12–14] Since the introduction of fluoroquinolones for ophthalmic use, the reported incidence of in vitro resistance to fluoroquinolones in bacteria isolated from cases with bacterial keratitis and endophthalmitis has been steadily increasing. A previous study reviewed the database of bacterial flora cultured from the conjunctival sac of 1455 Japanese patients scheduled for intraocular surgeries between 1995 and 2002.[14] The incidence of in vitro resistance of bacterial isolates to ofloxacin increased from 13.5% in 1995 to 32.8% in 1999. Moreover, when ofloxacin was replaced by LVFX in 2000, the incidence of resistance to LVFX gradually increased from 14.5% in 2000 to 20.5% in 2002.

The primary targets of fluoroquinolones are two essential enzymes of bacterial cells, DNA gyrase and topoisomerase IV.[15–17] In *S epidermidis*, DNA gyrase is composed of the GyrA and GyrB subunits encoded by the *gyrA* and *gyrB* genes, respectively. Topoisomerase IV is composed of ParC and ParE subunits encoded by *parC* and *parE* genes, respectively. In most bacterial species, mutations occur in the highly conserved quinolone resistance-determining regions (QRDR) of the genes that encode DNA gyrase and topoisomerase IV. In *Staphylococcus aureus*, several studies have shown that a combination of mutations in both genes can cause high-level resistance even to the newer fluoroquinolones.[18–21] However, the prevalence of mutations in the QRDR in *S epidermidis* isolated from the ocular surface and its association with fluoroquinolone resistance have not been fully investigated.[15–17] The present study analysed mutations in the QRDR of *gyrA*, *gyrB*, *parC* and *parE* genes of 138 isolates of *S epidermidis* recovered from conjunctival flora. The susceptibility of these isolates to LVFX, GFLX, MFLX and TFLX was also determined.

Table 1 Primers used in the study. Nucleotide positions are indicated according to GenBank sequence number NC 002976 (*S epidermidis* RP62A)

Target gene	Primer sequence (5' to 3')	Product size (bp)	Position
gyrA	ATGCGTGAATCATTCTTAGACTATGC	284	2 609 699–2 609 724
	GAGCCAAAGTTACCTTGACC		2 609 441–2 609 460
gyrB	CAGCATTAGACGTTTCAAG	251	2 610 508–2 610 528
	CCAATACCCGTACCAAATGC		2 610 278–2 610 297
parC	TCGCAATGTATTCAAGTGGG	197	939 185–939 204
	ATCGTTATCGATACTACCATT		939 361–939 381
parE	AAGCTCAACAAGCACGCGAGGCTG	324	938 196–938 219
	TTAAAGTCAGTACCAACACCAGCAC		938 493–938 520

METHODS

Bacterial isolates and susceptibility testing

One hundred and thirty-eight isolates of *S epidermidis* were collected from the conjunctival sac of 138 eyes of 129 patients who were scheduled for intraocular surgery at the National Tokyo Medical Center between November 2004 and June 2005. The mean (SD) age of the patients was 70.7 (14.9) years (range 6–91 years). The patients had not received either ophthalmic or systemic antibiotics prior to bacterial sampling.

Scrapes of the inferior conjunctival fornix were taken in the absence of topical anaesthetic using a sterile cotton swab. The samples were immediately inoculated into Mueller-Hinton (MH) agar and incubated at 35°C in air for 16–20 h for the selection of staphylococci. The MicroScan WalkAway-96 (Baxter Japan, Tokyo) with MicroScan Rapid Pos Combo Panel (Baxter) was used for the identification of *S epidermidis*. Positive cultures were stored at −80°C until the agar dilution testing to determine the minimum inhibitory concentration (MIC).

MICs for LVFX, GFLX, MFLX and TFLX were determined by the agar dilution method in accordance with the recommendations of the Japanese Society of Chemotherapy.[20] The bacterial suspensions in saline were inoculated on MH agar plates supplemented with defined concentrations of drugs. The plates were incubated at 35°C under aerobic conditions and MICs were determined after 20–24 h of incubation. Drug concentrations ranged from 0.025 μg/ml to 100 μg/ml in twofold increments except for TFLX (0.025 μg/ml to 25 μg/ml) because of its limited solubility.

DNA amplification and sequencing of QRDR

The isolates were suspended in tryptic soy broth and cultured overnight. Genomic DNA was extracted using the Wizard SV 96 genomic DNA purification system (Promega KK, Japan). One μl of the genomic DNA solution was applied in 20 μl of amplification mixture (5 pM each primer, 1.6 μl dNTP mixture, 2 μl Ex Taq buffer and 0.1 μl LA Taq (Takara Bio Inc, Japan)). Polymerase chain reaction (PCR) amplification was performed with the primers as shown in table 1. PCR primers were selected from the published sequences of *S epidermidis* RP62A. Each reaction was amplified with the following temperature profiles: 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. The amplified DNA products were separated and identified by 2% agarose gel electrophoresis.

PCR products were purified using ExoSAP according to the manufacturer's instructions (GE Healthcare Bio-Sciences KK, Japan). PCR-amplified DNA was sequenced by the dye

Table 2 Mutations in the quinolone resistance determining regions of *gyrA*, *parC* and *parE* in 70 strains of *Staphylococcus epidermidis*

Mutation type	No of isolates	Mutation			
		gyrA	*gyrB*	*parC*	*parE*
1	28	Ser84Phe	–	Ser80Tyr	–
2	1	Ser84Phe	–	Ser80Tyr + Asp84Val + Ala85Ser	–
3	4	Ser84Phe	–	Ser80Phe	–
4	4	Ser84Phe	–	Ser80Phe + Asp84Tyr	–
5	1	Ser84Phe	–	Ser80Phe + Asp84Asn	–
6	2	Ser84Phe	–	Asp84Tyr	–
7	5	Ser84Tyr	–	Ser80Phe	–
8	3	Ser84Tyr	–	Ser80Phe	Asp434Asn
9	1	Ser84Tyr + Glu88Lys	–	Ser80Phe + Asp84Ala	–
10	2	Ser84Tyr	–	Ser80Tyr	–
11	1	Ser84Tyr	–	Ser80Ile	–
12	1	Ser84Ile	–	Ser80Phe	Asn404Ser + Asp434Asn
13	1	–	–	Ser80Tyr	Asn404Ser
14	1	–	–	Ser80Phe	–
15	1	–	–	Asp69Asn	–
16	1	–	–	Ser81Pro	–
17	1	–	–	Asp84Gly	–
18	11	–	–	–	Asn404Ser
19	1	–	–	–	Asp434Asn

Table 3 Susceptibility of strains *of S epidermidis* to four fluoroquinolones

	No of isolates with the following MIC (μg/ml)												
	0.025	0.05	0.1	0.2	0.39	0.78	1.56	3.13	6.25	12.5	25	MIC_{50}	MIC_{90}
All isolates (n = 138)													
LVFX			26	55	4		23	19	7	3	1	0.2	3.13
GFLX		29	52	4	1	24	24	2	1	1		0.1	1.56
MFLX	1	72	9	3	26	16	7	3		1		0.05	0.78
TFLX	35	47	3		2	21	10	10	8	2		0.05	3.13
Wild type (n = 68)												Mode	
LVFX			24	44								0.2	
GFLX		26	42									0.1	
MFLX	1	60	7									0.05	
TFLX	30	38										0.05	
Mutations in *parC* and/or *parE* (n = 17)												Mode	
LVFX			1	11	4			1				0.2	
GFLX		2	10	4			1					0.1	
MFLX		11	2	3		1						0.05	
TFLX	4	9	3			1						0.05	
Mutations in both *gyrA* and *ParC* (n = 53)												Mode	
LVFX			1				23	18	7	3	1	1.56	
GFLX		1			1	24	23	2	1	1		0.78	
MFLX		1			26	15	7	3		1		0.39	
TFLX	1				2	20	10	10	8	2		0.78	

GFLX, getifloxacin; LVFX, levofloxacin; MFLX, moxifloxacin; TFLX, tosufloxacin.

terminator method in both the forward and reverse directions. Using Phred/Phrap/Polyphred software, the quality score of each base was calculated. Sample sequences were compared with a reference sequence and mutations were detected. The strain *S epidermidis* ATCC 35984 (RP62A) was used as a reference.

RESULTS

The mutations identified in the QRDR of the *gyrA*, *gyrB*, *parC* and *parE* genes are summarised in table 2. Nineteen different combinations of mutations were identified in 70 isolates, whereas no mutations were detected in 68 isolates. Three isolates (mutation profile type 2, 9 and 12) had four amino acid substitutions, 8 isolates (mutation profile type 4, 5 and 8) had three amino acid substitutions, 43 isolates (mutation profile type 1, 3, 6, 7, 10, 11 and 13) had double amino acid substitutions and 16 isolates (mutation profile type 14–19) had single amino acid substitutions.

In the *gyrA* gene, a single-point mutation was found in 53 isolates at codon 84. Double-point mutations in the *gyrA* gene were identified in 1 isolate at codons 84 and 88 (mutation profile type 9). No mutations were found in the QRDR area of the *gyrB* gene. In the *parC* gene, single-point mutations were found in 51 isolates at codons 69, 80, 81, 84 or 85. Double-point mutations were identified in 6 isolates at codons 80 and 84 (mutation profile type 4, 5 and 9). Triple-point mutations were identified in 1 isolate at codons 80, 84 and 85 (mutation profile type 2). In the *parE* gene, single-point mutations were found in 16 isolates at codon 404 or 434. Double-point mutations were identified in 1 isolate at codons 404 and 434.

The MICs of the four tested fluoroquinolones against *S epidermidis* are shown in table 3. All four fluoroquinolones had a bimodal distribution in all isolates (n = 138). Isolates with no mutations in the QRDR (wild type, n = 68) were susceptible to fluoroquinolones. The modes (the number that appears the most) were 0.2 μg/ml for LVFX, 0.1 μg/ml for GFLX, 0.05 μg/ml for MFLX, and 0.05 μg/ml for TFLX. Isolates with mutations restricted in the QRDR of *parC* and/or *parE* (n = 17) showed similar susceptibilities to fluoroquinolones as wild type strains except for one strain with mutation profile type 18. The modes were 0.2 μg/ml for LVFX, 0.1 μg/ml for GFLX, 0.05 μg/ml for MFLX and 0.05 μg/ml for TFLX. Isolates with mutations in the QRDR of both *gyrA* and *parC* (n = 53) were less susceptible to fluoroquinolones. The modes were 1.56 μg/ml for LVFX, 0.78 μg/ml for GFLX, 0.39 μg/ml for MFLX and 0.78 μg/ml for TFLX. Of these 53 isolates, 51 had amino acid substitutions at GyrA84 and ParC80. One isolate (mutation profile type 9) with two amino acid substations both in GyrA and ParC had the highest MICs (25 μg/ml for LVFX, 12.5 μg/ml for GFLX, MFLX and TFLX, respectively).

DISCUSSION

The primary targets of fluoroquinolones are two essential enzymes of bacterial cells, DNA gyrase and topoisomerase IV.[18–20] In most bacterial species the mutations in the genes that lead to fluoroquinolone resistance are limited to a few point mutations at restricted positions of the genes called QRDR. The present study revealed that approximately half (50.7%) of *S epidermidis* isolates in the human conjunctival flora have mutation(s) in the QRDR area of *gyrA*, *gyrB*, *parC* and *parE* genes.

Fluoroquinolone resistance has been studied intensively in *S aureus*.[18–21] The genes encoding topoisomerase IV in *S aureus* are called *grlA* and *grlB*, which are analogous to *parC* and *parE* in *S epidermidis*, respectively. Fluoroquinolone resistance in *S aureus*

is generally associated with two single-point mutations in *gyrA* at codon 84, and in *grlA* at codon 80 or 84. *S aureus* isolates with higher levels of resistance are associated with the second mutation in *grlA* at codon 80 or 84, depending on the position of the first mutation. When the second mutation in *gyrA* occurs at codon 85 or 88, in addition to the first mutation at codon 84, the strain shows the highest fluoroquinolone resistance even to newer fluoroquinolones.[21]

The present QRDR sequencing results indicate that the major mechanism of fluoroquinolone resistance in *S epidermidis* is analogous to that of *S aureus*. Isolates with mutations restricted to the QRDR of *parC* and/or *parE* (n = 17) in this study were similarly susceptible to fluoroquinolones as wild type strains. However, the presence of two mutations (n = 53) in both *gyrA* gene (located at codon 84) and *parC* gene (located at codon 80) have been found to be associated with the development of fluoroquinolone resistance.[15 16]

In this study only one isolate (mutation profile type 9), which was highly resistant to all four fluoroquinolones tested, had two amino acid substitutions both in GyrA and ParC. Previous studies have shown that isolates of *S epidermidis* and *S aureus* with two amino acid substitutions both in GyrA and ParC (GrlA in *S aureus*) have the highest fluoroquinolone resistance. The isolates with this mutation type are reported to be relatively rare in *S epidermidis*[15 16] and to account for less than 10% in *S aureus*.[18–20] However, a high prevalence (50%) of two amino acid substitutions in both GyrA and GrlA has recently been reported.[21] The empirical use of newer fluoroquinolones without a proper clinical indication may produce additional resistant strains of *S epidermidis*, as has already occurred with *S aureus*.

One possible limitation of the present study was that the patients were scheduled for intraocular surgery. Bacterial isolates therefore represent conjunctival flora rather than ocular pathogens. However, in common ocular infections such as bacterial conjunctivitis and bacterial keratitis, pathogens are frequently the normal bacterial flora that reside on the ocular surface.[2–4] This is true even in cases of postoperative endophthalmitis, in which *S epidermidis* is the most common bacterial isolate from vitreous aspirates.[7 8] Organisms isolated from the vitreous were genetically identical to those collected from the ocular surface in 68–82% of patients with post-operative endophthalmitis,[7] suggesting that the study of in vitro susceptibility to various fluoroquinolones is valid.

Drug resistance is a serious concern in treating ocular infections. The current study showed that approximately half the *S epidermidis* isolates from the conjunctival flora have mutation(s) in the QRDR. Both *gyrA* gene and *parC* gene are associated with the development of fluoroquinolone resistance.

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Competing interests: None.

Ethics approval: The principles of the World Medical Association Declaration of Helsinki were followed. Each subject received a thorough explanation of the purpose of the study and all procedures involved in the study, and provided written informed consent prior to enrolment. Approval for this investigation was granted by the Committee for the Protection of Human Subjects at National Tokyo Medical Center.

REFERENCES

1. **O'Brien TP.** Bacterial keratitis. In: Krachmer JH, Mannis MJ, Holland EJ, eds. *Cornea and external disease: clinical diagnosis and management*. Volume II. St Louis: Mosby-Year Book, 1997:1139–90.
2. **Greves A,** Henry M, O'Brien TP, *et al*. In vitro susceptibilities of bacterial ocular isolates to fluoroquinolones. *Cornea* 2001;**20**:301–5.
3. **O'Brien TP,** Maguire MG, Fink NE, *et al*. Efficacy of ofloxacin versus cefazolin and tobramycin in the therapy for bacterial keratitis. Report from the Bacterial Keratitis Study Research Group. *Arch Ophthalmol* 1995;**113**:1257–65.
4. **Study Group of National Surveillance of Infectious Keratitis in Japan.** National surveillance of infectious keratitis in Japan. Current status of isolates, patient background, and treatment. *J Jpn Ophthalmol Soc* 2006;**110**:961–72.
5. **Frohlich SJ,** deKaspar HM, Grabson T, *et al*. Bacterial keratitis in patients with and without contact lens anamnesis. *Klin Monat Augenheilk* 1999;**214**:211–6.
6. **Bourcier T,** Thomas F, Borderie V, *et al*. Bacterial keratitis: predisposing factors, clinical and microbiological review of 300 cases. *Br J Ophthalmol* 2003;**87**:834–8.
7. **Han DP,** Wisniewski SR, Wilson LA, *et al*. Spectrum and susceptibilities of microbiologic isolates in the Endophthalmitis Vitrectomy Study. *Am J Ophthalmol* 1996;**122**:1–17.
8. **Sandvig KU,** Dannevig L. Postoperative endophthalmitis: establishment and results of a national registry. *J Cataract Refract Surg* 2003;**29**:1273–80.
9. **Neu HC.** Microbiologic aspects of fluoroquinolones. *Am J Ophthalmol* 1991;**112**(Suppl 4):S15–24.
10. **Leibowitz HM.** Clinical evaluation of ciprofloxacin 0.3% ophthalmic solution for treatment of bacterial keratitis. *Am J Ophthalmol* 1991;**112**(Suppl 4):S34–47.
11. **Hyndiuk RA,** Eiferman RA, Caldwell DR, *et al*. Comparison of ciprofloxacin ophthalmic solution 0.3% to fortified tobramycin-cefazolin in treating bacterial corneal ulcers. *Ophthalmology* 1996;**103**:1854–62.
12. **Chelitz MG,** Hofling-Lima AN, Paranhos A, *et al*. Shifting trends in in vitro antibiotic susceptibilities for common ocular isolates during period of 15 years. *Am J Ophthalmol* 2004;**137**:43–51.
13. **Goldstein MH,** Kowalski RP, Gordon YJ. Emerging fluoroquinolone resistance in bacterial keratitis. *Ophthalmology* 1999;**106**:1313–8.
14. **Kurokawa H,** Hayashi K, Konishi M, *et al*. Increasing ofloxacin resistance of bacterial flora from conjunctival sac of preoperative ophthalmic patients in Japan. *Jpn J Ophthalmol* 2002;**46**:586–9.
15. **Dubin DT,** Fitzgibbon JE, Nahvi MD, *et al*. Topoisomerase sequences of coagulase-negative staphylococcal isolates resistant to ciprofloxacin or trovafloxacin. *Antimicrob Agents Chemother* 1999;**43**:1631–7.
16. **Li Z,** Deguchi T, Yasuda M, *et al*. Alteration in the GyrA subunit of DNA gyrase and the ParC subunit of DNA topoisomerase IV in quinolone-resistant clinical isolates of Staphylococcus epidermidis. *Antimicrob Agents Chemother* 1998;**42**:3293–5.
17. **Sreedharan S,** Peterson LR, Fisher LM. Ciprofloxacin resistance in coagulase-positive and -negative staphylococci: role of mutations at serine 84 in the DNA gyrase A protein of Staphylococcus aureus and Staphylococcus epidermidis. *Antimicrob Agents Chemother* 1991;**35**:2151–4.
18. **Weng T,** Tanaka M, Sato K. Detection of grlA and gyrA mutations in 344 Staphylococcus aureus strains. *Antimicrob Agents Chemother* 1998;**42**:23–40.
19. **Hooper DC.** Fluoroquinolone resistance among Gram-positive cocci. *Lancet Infect Dis* 2002;**2**:530–8.
20. **Horii T,** Suzuki Y, Monji A, *et al*. Detection of mutations in quinolone resistance-determining regions in levofloxacin- and methicillin-resistant Staphylococcus aureus: effect of the mutations on fluoroquinolone MICs. *Diagn Microbiol Infect Dis* 2003;**46**:139–45.
21. **Iihemi H,** Suzuki T, Kawamura Y, *et al*. Emerging multiple mutations and high-level fluoroquinolone resistance in methicillin-resistant Staphylococcus aureus isolated from ocular infections. *Diagn Microbiol Infect Dis* 2006;**56**:297–303.
22. **Japanese Society of Chemotherapy.** Revision of methods for determining minimum inhibitory concentrations. *Chemotherapy* 1981;**29**:76–9.

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Conjunctival Fixation Sutures for Refractory Superior Limbic Keratoconjunctivitis

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Case Report

A 75-year-old Japanese woman was referred to us for persistent irritation in September 2006. She reported a history of foreign body sensation in the left eye for 4 years. Slit lamp examination revealed hypertrophy, hyperemia, and pronounced fluorescein staining of the superior bulbar conjunctiva in the left eye (Figure 1-A, B). Schirmer's test results were 2 mm in the right eye and 1 mm in the left eye. The diagnosis of superior limbic keratoconjunctivitis (SLK) with aqueous deficiency dry eye was made based on these findings. Initially, treatment was attempted with 0.1% fluorometholone and 0.1% sodium hyaluronate eyedrops without success. Punctal plugs inserted to the upper and the lower puncta provided little symptomatic relief.

We applied fixation sutures to the superior bulbar conjunctiva in December 2006 to attempt to treat this refractory condition. First, the patient received a topical and subconjunctival injection of lidocaine 2%. A traction suture was made by placing a 6-0 silk suture at the limbus at the 12 o'clock position, so as to rotate the eye downward (Figure 2). Stretching the redundant superior bulbar conjunctiva with a spatula, anchoring sutures were then placed using 10-0 nylon sutures at a location 10-12 mm from the limbus to fixate the conjunctiva with the sclera. Two stitches were placed nasally from the superior rectus muscle, and three stitches were placed temporally. Postoperatively, the patient received 0.5% levofloxacin and 0.1% fluorometholone eyedrops four times daily for 2 weeks. Two weeks after the procedure, the patient reported complete resolution of symptoms. Slit-lamp examination revealed a marked reduction in conjunctival hyperemia and fluorescence staining of the affected area (Figure 1-C,D). The patient's findings remained stable through over one year of follow-up.