図2 Goldenhar症候群にみられる副耳
（後藤　浩先生のご厚意による）

図3 デルモリポーマと輪部デルモイドが連続してみられた例
輪部デルモイドの奥，結膜嚢側にデルモリポーマがみられる．

図4 眼窩脂肪ヘルニア
眼窩脂肪が球結膜下に脱出したものであり，柔らかく可動性があることで鑑別できる．

□鑑別の対象となる腫瘍

先天性の病変であり，特徴的な外観から診断は通常容易である．成人例で時に鑑別を要するのは眼窩脂肪ヘルニアである（図4）．上耳側の球結膜に隆起性病変がみられる点で類似するが，高齢者の男性に多い疾患であること，両眼性の場合が多いこと，隆起性病変は柔らかく可動性があること（スパーテルなどで触ると結膜下の脂肪織を奥に押し込むことができる）などから鑑別できる．

（山田昌和）

One Point Advice

保存角膜を利用した輪部デルモイドの治療

国立病院機構東京医療センター感覚器センター 山田昌和

手術をしても視機能は向上しない

輪部デルモイドでは角膜乱視，遠視性不同視を伴うことが多く，2/3の症例で不同視弱視を合併する．手術をしても角膜乱視は軽減されず，不同視も解消されないので，視力の向上は期待できない．手術はあくまで整容的なものと考えておくべきである．

幼少児では術後の炎症反応が強く，術後管理もむずかしいことから，原則的に低年齢のうちは手術は行わない．まず，眼鏡による屈折矯正と健眼遮閉を軸とした弱視治療を優先し，視力がある程度安定した時期，4～6歳くらいで手術を考慮するとよい．

保存角膜を用いた表層角膜移植

輪部デルモイドの場合，腫瘍切除だけでは病変の切除が不完全になりやすく，術後に強い炎症反応をきたすことがある(図1)．ほとんどの場合，デルモイドは角膜実質の半分くらいにまで達しているので，表層角膜移植を前提として手術を計画した方がよい．ドナー角膜は新鮮角膜である必要はなく，冷凍またはグリセリンで保存された角膜でよい．

輪部デルモイドにトレパンを当てて，腫瘍全体を切除できる最小の大きさのサイズを選択する．また，切除縁や縫合糸ができるだけ瞳孔領にかからないように配慮する(図2, 3)．トレパンで半層程度角膜を切開し，層間剝離を進めていく．強膜側はあまり深く切除する必要はないが，角膜側は混濁が消えるレベルまで層間剝離を繰り返して，できる限り完全切除を心がける．

移植片はレシピエントと同サイズのトレパンと人工前房装置を用いて作成する．保存角膜は膨潤しているので少し厚めに作った方が良い．縫合は10-0ナイロンの端々縫合で行い，大体12針程度とする．

術後は抗菌薬とステロイド薬の点眼を行う．糸が緩んでくると糸周囲に白色浸潤が生じるので，年長児では緩んだ糸から抜糸していく．だいたい5～6歳以降であるとスリット下で抜糸可能なことが多い．幼少児など外来での抜糸が不可能な場合には，術後2～3ヵ月に全身麻酔下で全抜糸を行う．抜糸は早め早めにしたほうが，術後の経過が良いようである．

[図1] 輪部デルモイドの術後の例
表層切除だけで対処したが，病変の残存があり，強い炎症反応を生じている．

[図2] 輪部デルモイドの術前．

[図3] 輪部デルモイドの表層移植後
図2と同じ症例の術後．切除縁や縫合糸ができるだけ瞳孔領にかからないようにする．

感染性角膜炎にステロイドは禁忌だろうか

山田 昌和（国立病院機構東京医療センター感覚器センター）

感染性疾患の治療は，病原微生物の同定と病原微生物に効力をもつ抗菌薬，抗真菌薬の投与が基本であることはいうまでもない．感染性角膜炎の場合に問題となるのは，病態に2つの要素があり，病原微生物による組織の直接的な損傷だけでなく，生体反応として浸潤した白血球や活性化された角膜実質細胞による二次的な組織損傷を考慮しなければならない点である．皮膚など他の組織では瘢痕治癒でかまわないが，角膜の場合は瘢痕性混濁や角膜形状異常は視機能低下に直結してしまう．組織破壊を最小限にとどめたうえで，病原微生物を排除するバランスが要求されることになる．

ここで，ステロイド投与の是非が問題となる．この問題は以前から議論があり，必ずしも一致した見解が得られていない．この問題についてもう一度考えてみよう．

ステロイドは感染の誘因となり予後に悪影響を与える

角膜移植眼などでステロイドの点眼を長期に用いていると，感染性角膜炎が誘発されやすいことはよく知られている．宿主の免疫能が抑制されるために感染が成立しやすくなると考えられ，動物モデルによる緑膿菌角膜炎モデルなどでも実証されている．臨床的にも，Luchs らは水疱性角膜症に発生した感染性角膜炎について retrospective に調査し，ステロイド点眼によって発症のリスクが2.63倍に上昇することを示している[1]．水疱性角膜症では，角膜上皮の脆弱性や治療用コンタクトレンズ（CL）なども発症に関係するものと推測されるが，ステロイドが感染性角膜炎発症の誘因になることは間違いなさそうである．

ステロイドを用いていた症例では感染性角膜炎の予後が不良であることも示されている．Wilhelmus のメタアナリシスによれば，感染性角膜炎の発症前にステロイドを用いていた症例では，治療が不成功（角膜穿孔や眼内炎など）に終わる場合が多く，その相対危険率は3.59（95％信頼区間2.42～5.35）と報告されている[2]．選択バイアス（ステロイドがもともと使われている症例には角膜基礎疾患がある場合が多い）も考慮すべきであるが，メタアナリシスのもとになった6つの一次研究の結果もほぼ一致していることから，この結論は妥当と考えてよいものと思われる．

ステロイドは感染性角膜炎の臨床像を変えてしまう

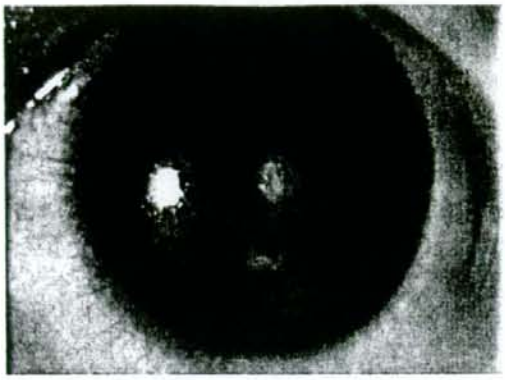
ステロイドが使われている症例に感染性角膜炎が発症した場合に留意すべき点は，ステロイドが角膜感染症としての臨床像を変えてしまう点である．通常，われわれが診る感染性角膜炎の臨床像は，病原微生物による直接侵襲と，これに対する生体反応，炎症反応が合わさって成り立っている．

❶にCL装用者に生じた表皮ブドウ球菌による角膜炎，❷にフリクテン性角膜炎でステロイド点眼中に生じたカンジダ角膜炎の写真を示す．どちらも感染性角膜炎であるが，両者の所見は大きく異なっている．❶では細菌の直接の浸潤巣は小さいのに，周囲の角膜には浮腫があり，強い充血と前房内炎症を伴っており，患者も強い痛みを訴える．直接の浸潤巣以外は生体反応の結果といえるかも知れない．一方，❷では，白色の浸潤巣は❶より大きいのに，これに対応する炎症反応がほとんどみられない．このため，当初は感染ではなく，なんらかの沈着物と考えていたほどであった．ステロイドが角膜感染症の臨床像を変えてしまう例であり，診断や治療の間違いや遅れが生じる可能性がある．

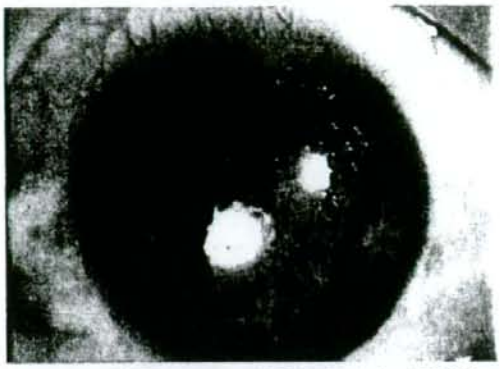
ステロイドは感染性角膜炎の治療に有用だろうか

次に，感染性角膜炎の治療経過のなかでステロイドを使うことの是非について考えてみたい．実験的な感染性角膜炎で抗菌薬と併用したステロイドの効果を検討した24編の論文（3編は肺炎球菌，2編は黄色ブドウ球菌，19編は緑膿菌）のうち，9編はステロイドが有用，3編は有用でない，12編は中立という結果であったという[2]．

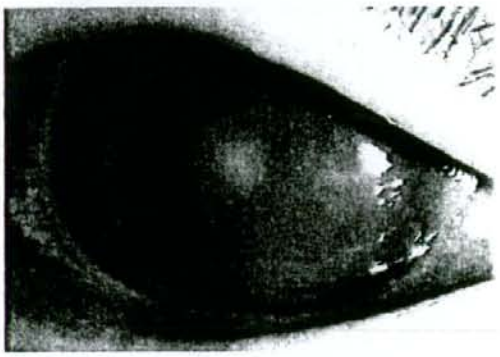
臨床的にはどうかというと，Wilhelmus は感染性角膜炎の治療中にステロイドを使用した場合の予後をメタアナリシスで評価している[2]．結果は，相対危険率は0.65（95％信頼区間0.38～1.11）で，ステロイドは有用とも無用ともいえないというものであった．臨床的エビデンスという観点からは，予後，合併症，罹病期間のいずれにおいてもステロイドの有



❶ 表皮ブドウ球菌による角膜炎
浸潤巣周囲の角膜に浮腫があり，強い充血と前房内炎症を伴っている．



❷ フリクテン性角膜炎でステロイド点眼中に生じたカンジダ角膜炎
白色の浸潤巣に対応する炎症反応がほとんどみられない．



❸ 緑膿菌による感染性角膜炎
輪状膿瘍を呈しており，角膜実質の菲薄化が危惧される．

用性は確認されていない．

ただし，こうした臨床研究が可能ということは，感染性角膜炎の治療経過でステロイドを使う場面が少なくないことを示している．ステロイドには，①痛みや充血など自覚症状の軽減に役立つ，②過剰な生体反応，炎症反応による二次的な組織損傷を軽減する，③matrix metalloproteinase 活性を阻害することにより実質の融解を防止する，などの効果が期待できるからである．

しかし実際には，感染性角膜炎に最初からステロイドを用いることは決して推奨されない．ステロイドを用いるにあたっての原則として Stern らは，①起因菌が同定されていること，②起因菌に有効な抗菌薬が投与されていること，③抗菌薬の有効性が臨床的に明らかであること，の3つをあげている[3]．投与している抗菌薬の臨床的効果が明らかとなった感染性角膜炎の治りかけの時期に，自覚症状の軽減や罹病期間の短縮，合併症の防止のために後からステロイドを加えるということになる．❸は CL 装用者に生じた緑膿菌による角膜潰瘍の症例である．角膜実質の融解による菲薄化や混濁の残存が危惧される症例であり，この症例ではアミノ配糖体系抗菌薬の頻回点眼による臨床症状の改善を確認した後に，ステロイドの点眼を追加した．

細菌に比べて，真菌やアメーバでは抗真菌薬など治療薬の効果が出にくいために，ステロイドの使用は慎重に行うべきとされている[3]．とくに真菌の場合には，ステロイドの使用は原則的に禁忌であるという考え方が一般に支持されている．アメーバの場合には，ステロイドを使ってもよいという意見も使うべきでないとする意見もあり，評価は分かれている．

以上のように，感染性角膜炎の治療におけるステロイドの役割は結論の出ていない問題である．個人的には，Stern らの原則を守りつつ，感染性角膜炎の治りかけの時期に用いると自覚症状の軽減，罹病期間の短縮，合併症の防止などに役立つ場合があると考えている．

文献

1) Luchs JI, et al. Ulcerative keratitis in bullous keratopathy. Ophthalmology 1997；104：816-22.
2) Wilhelmus KR. Indecision about corticosteroids for bacterial keratitis. An evidence-based update. Ophthalmology 2002；109：835-44.
3) Stern GA, Buttross M. Use of corticosteroids in combination with antimicrobial drugs in the treatment of infectious corneal disease. Ophthalmology 1991；98：847-53.

ドライアイの診断には golden standard がない

山田 昌和（国立病院機構東京医療センター感覚器センター）

ドライアイは比較的新しい疾患概念であり，診断のための golden standard が存在しない．ドライアイの定義やドライアイという疾患概念に包括される内容も時代とともに変化してきており，2006 年から 2007 年にかけて，国内でも世界的にもドライアイの定義や診断基準の見直しがなされた（島崎潤［ドライアイ研究会］．あたらしい眼科 2007；24：181-4，Lemp MA, et al. Ocular Surface 2007；5：75-92）．ここでは日本の診断基準をもとにドライアイの診断についての問題点を考えてみたい．

新しい診断基準とドライアイ検査の不確実性

2006 年に改定されたドライアイ研究会によるドライアイの定義は，「様々な要因による涙液および角結膜上皮の慢性疾患であり，眼不快感や視機能異常を伴う」となっている．同時に改定された診断基準を❶に示す．

一見，わかりやすい定義と診断基準のようだが，ドライアイに使われる臨床検査の不確実性と，ドライアイでの自覚症状と検査結果との不一致のために臨床上はさまざまな問題が生じる可能性がある．

ドライアイに使われる臨床検査の不確実性とは，それぞれの検査の感度と特異度が十分ではないという意味である．感度は陽性者を正しく陽性と判定できる割合，特異度は陰性者を陰性と正しく判定できる割合である．例えば，Schirmer 試験については，感度は 10％から 85％，特異度は 68％から 100％と報告により幅があるが，これはカットオフ値（正常と異常をどこで区切るか）によって感度や特異度が変わってくるためである．

仮にドライアイの有病率を 10％として，ドライアイ研究会の診断基準に沿って 1,000 名に検査を行った場合を想定してみる．各検査の感度と特異度を代表的な報告から，自覚症状（感度 98％，特異度 97％），Schirmer 試験（感度 85％，特異度 83％），涙液層破壊時間（BUT：感度 72％，特異度 62％），生体染色試験（感度 77％，特異度 90％）とする．この値は報告のなかでできるだけ良い数字を採用するようにしている．診断基準の確定例に相当する症例がどうなるか，シミュレーションした結果を❷に示す．1,000 名のうち，ドライアイ患者は 100 名のはずであるが，ドライアイ確定例と診断されるのは 165 名となる．このなかにはドライアイ患者が 75 名含まれているが，残りの 90 名は正常者であり，陽性適中率はわずか 45％である．診断基準全体の感度は 75％，特異度は 90％と計算される．

ある疾患の診断を行うのに検査を組み合わせるのは良い方法のように一見思えるが，実際には検査を組み合わせても極端に感度や特異度を上昇させることはできないのである．ドライアイに限らず，golden standard がない疾患の診断は常にこの問題を内包している．

ドライアイの自覚症状と検査所見は一致しない？

ドライアイはある意味で自覚的な疾患である．ドライアイでは自覚症状の強さと検査所見が一致しないことは以前から知られていたが，最近は自覚症状を定量的に評価できる質問票を用いた研究がさかんに行われている．これらによるとドライアイの自覚症状は検査所見とあまり相関せず，検査の再現性は自覚症状調査よりも低いという（Nichols KK, et al. Cornea 2004；23：272-85，Nichols KK, et al. Cornea 2004；23：762-70）．

自覚症状に診断を頼るのは「科学的でない」と言われそうだが，必ずしもそうではない．鎮痛薬や片頭痛の治療薬は自覚症状をほぼ唯一の評価基準として臨床試験が行われている．ドライアイの診断や治療効果の判定に用いることのできる質問票も米国では開発されてきており，自覚症状を科学的に定量的に評価することは十分可能なのである．

「目が乾く，ごろごろする」という自覚症状だけでドライアイと診断することには問題があるが，自覚症状に目を向ける態度が，ドライアイを扱う臨床医には必要と思われる．

ドライアイの概念の拡がり

前述したドライアイの定義によると，ドライアイはさまざまな要因による眼表面の慢性疾患であり，単純に涙液が欠乏している疾患ではない．Meibom 腺機能不全，瞬目不全，コンタクトレンズ装用，点眼薬や内服薬の副作用，VDT 作業

❶ ドライアイの診断基準

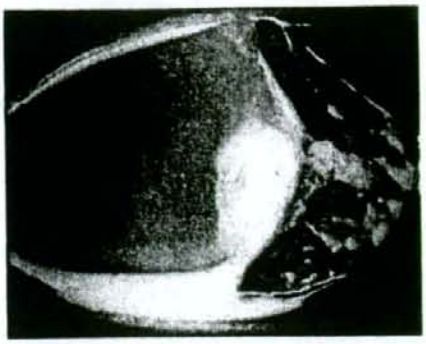
1. 自覚症状（視機能異常を含む）があること
2. 涙液の異常
   ①Schirmer 試験 I 法で 5mm 以下
   ②涙液層破壊時間（BUT）5 秒以下
   ①，②のいずれかを満たすものを陽性とする
3. 角結膜上皮障害
   ①フルオレセイン染色スコア 3 点以上（9 点満点）
   ②ローズベンガル染色スコア 3 点以上（9 点満点）
   ③リサミングリーン染色スコア 3 点以上（9 点満点）
   ①，②，③のいずれかを満たすものを陽性とする

1，2，3 のすべてを満たすものを確定例とする．
1，2，3 のうち 2 つを満たすものを疑い例とする．
ただし，1 と 3 の項目を満たす疑い例（涙液の異常を認めない角結膜上皮障害）の場合は，ドライアイ以外の原因検索を行うことを基本とする．

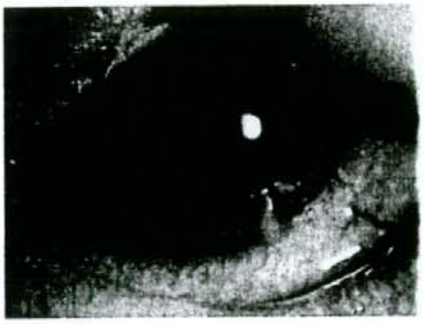
❷ ドライアイ診断基準の感度と特異度

	ドライアイと診断される数	正常と診断される数	合計
ドライアイ患者	75	25	100
正常者	90	810	900
合計	165	835	1,000

ドライアイの有病率を 10％と仮定し，1,000 名の集団をドライアイ研究会の診断基準に沿って検査を行った場合を想定した．診断基準全体の感度は 75％，特異度は 90％と計算される．



❸ 結膜下出血に続発した dellen
異所性メニスカスによって隣接する角膜周辺部の乾燥から dellen が生じたと考えられ，局所的ドライアイと考えることもできる．



❹ 片側顔面痙攣の症例に生じた糸状角膜炎
眼瞼痙攣による眼瞼と眼球の摩擦抵抗増大が発症に関与していると考えられ，相対的な潤滑油（涙液）不足によるドライアイと考えることもできる．

など環境要因によるもの，結膜弛緩症など，さまざまな病態が含まれ，極端に言えばアレルギー性結膜炎と感染症以外の慢性眼表面疾患はすべてドライアイに包括されることになる．実際，ドライアイのサブタイプ別頻度を調査した最近の報告では，最も頻度が高いのは Meibom 腺機能不全であり，涙液減少型のドライアイは 16.7％と少数派になっている（Albietz JM. Optom Vis Sci 2000；77：357-63）．涙液減少症もしくは乾性角結膜炎という今までの概念に比べて，ドライアイの疾患概念はかなり拡がっていることに注意したい．

ドライアイと呼んでよいかどうか境界領域の例を 2 つ呈示する．❸は結膜下出血に続発した dellen の例であり，❹は片側顔面痙攣の症例に生じた糸状角膜炎である．❸の症例では結膜の隆起によって異所性メニスカス（異常な涙液貯留部位）が生じており，隣接する角膜周辺部の乾燥によって dellen が生じたと解釈していくと，局所的なドライアイという病態になるし，❹は眼瞼痙攣により，眼瞼と眼球の摩擦抵抗増大が発症に関与していると考えられ，その基本的メカニズムは乾性角結膜炎で生じる潤滑油（涙液）不足による糸状角膜炎に類似していることがわかる．いずれの症例も局所的や相対的なものであるが，涙液の観点から病態をとらえると広い意味でのドライアイのカテゴリーに入るものと思われる．

入り口の診断名としてのドライアイ

ドライアイは慢性の眼表面疾患を総称する診断名と考えておいて，もう一歩先の診断名に踏み込むことが，診断や治療のうえで重要だと筆者は考えている．急性結膜炎だけでは診断名として不十分で，アレルギー性，細菌性，ウイルス性といったもう少し詳しい診断が必要なのと似ている．ドライアイの治療は人工涙液だけ，という時代は遠く過ぎ，さまざまな治療のオプションが選択できる現在，ドライアイという診断に満足しない姿勢がこれからのドライアイ診療には要求されるのではないだろうか．

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

H Mochizuki,[1] M Yamada,[1] S Hato,[1] T Nishida[2]

Division for Vision Research, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan; [2] Department of Ophthalmology, Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan

Correspondence to: M Yamada, Division for Vision Research, National Institute of Sensory Organs, National Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro, Tokyo 152-8902, Japan; yamadama-okazu-1@kakuki.go.jp

Accepted 6 June 2007

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. 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.

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, several studies have reported that hyaluronic acid is able to improve the symptoms, signs and ocular surface damage associated with dry eye syndrome. 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. Hyaluronic acid enhances water retention on the corneal surface, and probably increases corneal wettability. In addition, hyaluronic acid promotes migration of corneal epithelial cells and accelerates the healing of corneal epithelial defects.[4–6] 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.[7 8]

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.[9] 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 ^{99m}Tc as a tracer,[10] 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 hyaluronic 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.[2] 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.[2]

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. The residence time of topically applied hyaluronic acid has previously been investigated by using gamma scintigraphic methods.[1] Snibson and associates [2] reported that hyaluronic acid had prolonged ocular residence times in comparison

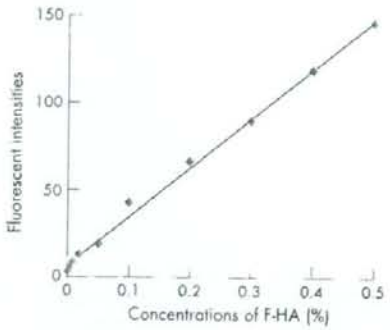


Figure 1 Calibration of the fluorescent intensities against the concentrations of fluorescein hyaluronic acid (F-HA). The relationship between the fluorescent intensities and the concentrations of F-HA was linear ($r^2 = 0.995$).

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 hyarulonic 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.[9–11] 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.[12 13]

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.[14–16] Snibson and associates[9] 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 23.3 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.[17 18]

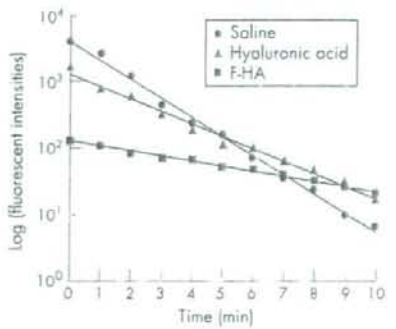


Figure 2 Typical result of the turnover rate measurements obtained from one subject. In the presented case, the fluorescent intensities of 0.1% flurorescein hyaluronic acid (F-HA) decayed with time at a flow rate of 7.6%/min, which was lower than those of fluorescein sodium in 0.1% hyaluronic acid (19.4%/min) 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.[1 2] 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.[19 20] 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. **Holly 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* 1980;**106**:484–7.
5. **Polack FM,** McNiece MJ. 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,** Anderson 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,** McCoy 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**:53–61.

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, Kinoal 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**, Pancher KS, Brun 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**, Horinoto 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**:978–37.
21. **Johnson ME**, Murphy PJ, Boulton M. Effectiveness of sodium hyaluronate eyedrops in the treatment of dry eye. *Graefe's Arch Clin Exp Ophthalmol* 2005;**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.

Eye & Contact Lens 34(2): 109–112, 2008

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

From the Division for Vision Research, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan.

Supported in part by a grant from the Ministry of Health, Labor, and Welfare, Tokyo, Japan.

Address correspondence and reprint requests to Dr. M. Yamada, Division for Vision Research, National Institute of Sensory Organs, National Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan; e-mail: yamadamasakazu@kankakuki.go.jp

Accepted June 11, 2007

DOI: 10.1097/ICL.0b013e31813c102a

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

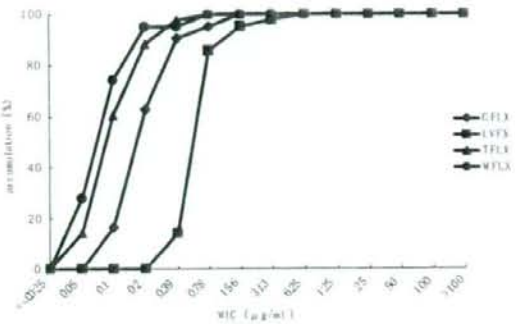


FIG. 1. The accumulation curves of the minimum inhibitory concentration (MIC) of the four fluoroquinolones against α-hemolytic streptococci (n = 43). Circle, moxifloxacin; triangle, tosufloxacin; diamond, gatifloxacin; square, levofloxacin.

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.

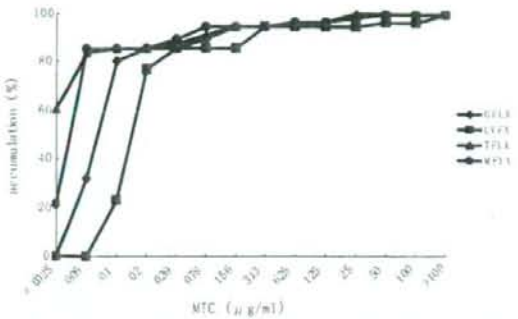


FIG. 2. The accumulation curves of the minimum inhibitory concentration (MIC) of the four fluoroquinolones against *Staphylococcus aureus* (n = 56). Triangle, tosufloxacin; circle, moxifloxacin; diamond, gatifloxacin; square, levofloxacin.

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

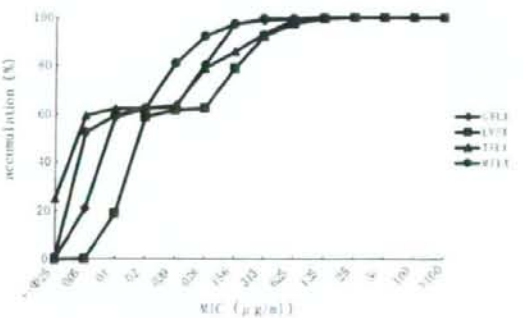


FIG. 3. The accumulation curves of the minimum inhibitory concentration (MIC) of the four fluoroquinolones against *Staphylococcus epidermidis* (n = 140). Triangle, tosufloxacin; circle, moxifloxacin; diamond, gatifloxacin; square, levofloxacin.

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.

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

M Yamada, J Yoshida, S Hatou, T Yoshida, Y Minagawa

Division for Vision Research, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan

Correspondence to: Dr M Yamada, Division for Vision Research, National Institute of Sensory Organs, National Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan; yamadamasakazu@kankakuki.go.jp

Accepted 10 April 2008

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_{90} 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.[22] 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, gatifloxacin; 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.[3–6] 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.

Funding: Supported in part by a grant from the Ministry of Health, Labour and Welfare, Japan.

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. **Graves 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,** 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.
13. **Goldstein MH,** Kowalski RP, Gordon YJ. Emerging fluoroquinolone resistance in bacterial keratitis. *Ophthalmology* 1999;**106**:1313–8.
14. **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**:586–9.
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. **Wang 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. **Ihara H,** Suzuki T, Kawamura Y, *et al*. Emerging multiple mutations and high-level fluoroquinolone resistance in methicilin-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.

Conjunctival Fixation Sutures for Refractory Superior Limbic Keratoconjunctivitis

Masakazu Yamada, M.D., Shin Hatou, M.D., and Hiroshi Mochizuki, M.D.

Division for Vision Research, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan

Correspondence:

Masakazu Yamada, M.D.

Division for Vision Research, National Institute of Sensory Organs,

National Tokyo Medical Center

2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan

Phone: +81-3-3411-0111 ext. 5236

FAX: +81-3-3411-0185

E-mail: yamadamasakazu@kankakuki.go.jp

Running head: Fixation Sutures for Superior Limbic Keratoconjunctivitis

Key words: conjunctivochalasis, cornea, dry eye, keratitis, superior limbic keratoconjunctivitis

Competing Interest: None declared.

Supported by a grant from the Ministry of Health, Labor, and Welfare of Japan.