表10　前回調査（文献[1]の原データから抽出）と今回調査の対象者の年齢比較

	前回調査（183例）	今回調査（年齢不明の４例除く165例）
最少年齢～最高年齢	16～54歳	16～63歳
平均年齢±標準偏差	28±７歳	32±10歳
30歳未満	122例	82例
30歳以上	61例	83例
40歳未満	172例	135例
40歳以上	11例	35例

口部の細菌汚染と調査時の平均気温の間に関連はみられなかったが，植田ら[12]は重症CL関連角膜感染症が８月をピークとする暑い時期に多発することを報告している。重症CL関連角膜感染症[1]ではCLケースからの菌検出頻度も，緑膿菌，グラム陰性桿菌（緑膿菌，コリネバクテリウム，セラチア除く），アカントアメーバ，セラチアの順であり，通常の使用者のSCLケース[13]のCNS，*Bacillus subtilis*，*Micrococcus*という順とは異なっている。通常の使用者を対象とした調査結果をCL関連角膜感染症に罹患するような使用者に当てはめることはできないのかもしれない。また，今回の調査によるMPS容器出口からの細菌検出率は19％と，前回の調査結果[11]の32％より低かった。検出菌も前回[3]多く検出されたのは，*Bacillus subtilis*，CNS，*Serratia marcescens*，そのほかの順であったが，今回はCNS，*Micrococcus*，*Bacillus subtilis*，そのほかの順（表４）と多少の違いがある。時期的には前回の調査期間は９～10月と，１～３月の２期間，今回の調査期間は７～１月まで連続した１期間である。前回と今回の細菌検出率，検出菌の違いの原因としては，この調査期間の違いに加えて，参加施設の差，単なるばらつきなどが考えられる。

いずれにせよ前回，今回の通常使用者を対象とした調査から得られた結論としては，MPS容器出口部の細菌汚染は使用開始後ごく早期に発生し，その後，変化なく続いていることになる。Sweeneyら[9]はSCL用生理食塩水について，実際にSCLケアに使用した群と，単にキャップを開け閉めしただけの群との間で細菌，真菌の検出率に差がないことを報告しており，汚染がケア用品容器，とくにキャップの操作によって生じていることを示唆している。またSweeneyら[9]は使用期間の違いによって汚染に差が生じないことも報告しており，これは今回の調査結果とも一致する。

今回の調査では若年者が使用したMPS容器に汚染の発生が多かった。これは若年者の方がMPS容器の出口部に手指で触れてしまいやすい，あるいは事前の手洗いが雑であるなどの事情を示すのかもしれない。また使用頻度が比較的低い例に汚染が多いことも示されたが，これは不定期使用のためにMPS容器の扱いが一定せず，不注意な操作を行いがちだからとも解釈できる。いずれも憶測にすぎないが，MPS容器の汚染は開封後の時間経過よりも，使用者の行動，MPS容器の取り扱い方によって生じると考えることが可能である。最も多く検出されたCNS，*Micrococcus*はともにヒトの常在菌であり，洗浄不十分な手指などによる接触によって汚染されたと考えるのが妥当であろう。

前回の調査[11]では年齢と細菌汚染の間に関連はみられなかった。これは前回の調査では高年齢の使用者が比較的少なかったため（表10）とも考えられるが，今回の調査結果にたまたま偏りがあった可能性もある。また，今回SCLの使用頻度が低い例に細菌汚染が多い結果が得られたが，Yungら[10]はSCL使用頻度の低い症例が使用していたSCL本体は汚染率が高いと報告しているものの，SCL保存容器，SCL消毒剤内容液については差を認めておらず，消毒剤容器出口の擦過検体は検査していない。消毒剤の汚染に関する既報[5-10]も年齢との関係は報告していない。年齢や使用頻度とMPS容器汚染の関係を明らかにするためには，更に多数の例を対象とした調査が必要であろう。また，MPSがなくなるまで使用させたC群では検体の回収率が54％とかなり低かった（表３）。このためC群の結果は使用者の実態に即していないおそれがある。このような調査では収集した検体がどこまで現実を反映しているかにも注意する必要がある。

前回，今回ともMPS容器出口部から高率に細菌が検出された。SCL消毒にMPSを使用している症例，とくに若年者や使用頻度の低い者に対しては，SCLケア開始前に手指をよく洗うとともに，MPS容器の出口部に不用意に接触しないよう指導する必要がある。また使用開始後間のないMPSにおいてもすでに細菌汚染が発生している可能性があるため，容器出口部をSCL保存容器内のMPS液面に触れさせないなどの注意を払う必要がある。

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(2013年7月1日受付)

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JOURNAL OF OCULAR PHARMACOLOGY AND THERAPEUTICS
Volume 30, Number 4, 2014

DOI: 10.1089/jop.2013.0204

Ocular Penetration and Efficacy of Levofloxacin Using Different Drug-Delivery Techniques for the Prevention of Endophthalmitis in Rabbit Eyes with Posterior Capsule Rupture

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Abstract

Purpose: To evaluate the effects of different drug-delivery techniques for levofloxacin (LVFX) in ocular penetration and the prevention of endophthalmitis using an aphakic rabbit model with posterior capsule rupture (PCR).
Methods: LVFX was administered to aphakic rabbit eyes with or without PCR using eye drops (EDs), subconjunctival injection (SCI), or intracameral (IC) injection. The concentration of the drug in the vitreous and aqueous humors was estimated at 2 h after injection. In another study, aphakic rabbit eyes with PCR were inoculated with *Enterococcus faecalis*, immediately followed by 0.5% LVFX ED, 0.5% moxifloxacin (MFLX) ED, LVFX IC (500 μg/0.1 mL), or IC saline. EDs were administered 0, 3, and 6 h after surgery. Changes on electroretinography (ERG) and intraocular bacterial growth were determined sequentially until 48 h after inoculation.
Results: The concentrations of LVFX at 2 h after IC were higher in the aqueous humor and the vitreous cavity of eyes with or without PCR, compared with EDs or SCI. Eyes treated with LVFX ED, MFLX ED, or IC saline showed a significantly greater reduction in b-wave amplitude on ERG at 48 h compared with eyes treated with LVFX IC. The number of bacteria recovered from the vitreous humor in eyes treated with IC LVFX at 48 h was significantly less than from eyes that received other treatments.
Conclusion: The LVFX IC was effective at suppressing endophthalmitis caused by *E. faecalis* in eyes with a PCR.

Introduction

BACTERIAL ENDOPHTHALMITIS is one of the most severe and sight-threatening complications of cataract surgery. The incidence of postoperative endophthalmitis has decreased (from 0.20% to 0.04%),[1,2] mainly because of the introduction of new surgical techniques.[3] However, an invariably sight-threatening infection is often resolved with substantial visual loss, and prophylaxis for endophthalmitis should be considered, given its pathogenesis. The external bacterial flora probably enters the anterior chamber through the surgical wound; in fact, contamination of the anterior chamber at the end of surgery has been noted in as many as 5.7%–21.1% of cases.[4–7] Moreover, bacterial migration from the anterior chamber to the posterior chamber is a key event in the progression of postoperative endophthalmitis; in the posterior segment, severe retinal damages may result. Rupture of the posterior capsule during surgery leads to a significantly higher incidence of postoperative endophthalmitis[8,9] because aqueous humor contaminants can readily access the vitreous cavity. Several procedures have been attempted to reduce bacterial contamination of the eye and to prevent endophthalmitis.[10] Some reports have shown that intracameral (IC) antibiotics are effective in the prevention of endophthalmitis.[11,12] Along with IC antibiotics, antibiotic eye drops (EDs) may be used to prevent endophthalmitis. Generally, topical antibiotics are administered immediately after surgery and on the following day as postoperative prophylaxis for endophthalmitis.

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Wallin *et al.*[13] reported that starting topical antibiotic administration on the day after surgery rather than on the day of surgery was associated with an increased risk of endophthalmitis. These results suggest that bacteria proliferate and express virulence factors as early as 1 day after surgery. Indeed, we previously reported that immediate postoperative prophylaxis using a moxifloxacin (MFLX) ophthalmic solution reduced the risk of *Enterococcus faecalis*-caused endophthalmitis in a rabbit model.[14] Further, Colleaux and Hamilton[15] demonstrated that prophylactic subconjunctival antibiotic injections at the conclusion of cataract surgery decreased the incidence of postoperative endophthalmitis.

Thus, antibiotic administration at the end of cataract surgery could be effective for the prevention of endophthalmitis. However, little is known about the efficacy of antibiotics in eyes with posterior capsule rupture (PCR), which is a major risk factor for endophthalmitis. Moreover, the penetration of antibiotics using different drug-delivery techniques into the anterior or posterior segment of eyes with or without a ruptured posterior capsule has not been well documented.

This study was designed to investigate the pharmacokinetics of levofloxacin (LVFX) in the anterior chamber or vitreous cavity after the use of various drug-delivery techniques and the efficacy of antibiotics for the prophylaxis of postoperative endophthalmitis in eyes with PCR.

Materials and Methods

Antibiotics and bacteria

A 0.5% LVFX ophthalmic solution (Cravit; Santen Pharmaceutical, Co., Ltd., Osaka, Japan) and 0.5% MFLX ophthalmic solution (Vigamox; Alcon Japan Ltd., Tokyo, Japan) were purchased from their respective manufacturers. The laboratory strain of *E. faecalis* OG1S, which produces a secretory protease, was used.[16] The minimum inhibitory concentrations (MICs) of LVFX and MFLX against OG1S were 2.0 and 0.5 μg/mL, respectively. The bacteria were grown in brain heart infusion (Difco Laboratories, Detroit, MI) broth for 18 h at 37°C and then washed twice with sterile physiological saline and resuspended in sterile physiological saline. The concentration of bacteria in the suspension was determined spectrophotometrically and then adjusted to $\sim 2 \times 10^5$ colony-forming units/mL (CFU/mL) with sterile physiological saline.

Animals

Female Japanese albino rabbits, weighing 2 kg each (Kitayama Labes Co. Ltd., Nagano, Japan), were maintained in accordance with Institutional Animal Care and Use Committee guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Laboratory Animals in Ophthalmic and Vision Research. All procedures involving rabbits were approved by the Committee of Animal Experimentation, Ehime University School of Medicine (Matsuyama, Japan).

The rabbits were anesthetized with an intramuscular injection of an equal mixture of 5% ketamine (Ketalar intramuscular, 500 mg; Sankyo Co., Ltd., Tokyo, Japan) and 2% xylazine (Selactar; Bayer Ltd., Tokyo, Japan) at 1 mL/kg for all procedures. The rabbits were euthanized with an overdose of pentobarbital sodium.

Lensectomy

A lensectomy was performed on both eyes as described previously.[16] A clear corneal incision was performed, and the lens was extracted with a Phacompo Phacoemulsificator (Santen Pharmaceutical Co., Ltd.) using balanced salt solution (BSS plus; Alcon, Fort Worth, TX) for irrigation, and the incision was sutured with 10-0 nylon. Intentional rupture of the lens capsule was made in some cases using phaco tips.

Intraocular penetration of LVFX

After lensectomy with or without intentional rupture of the posterior capsule, a single dose of 0.5% LVFX ophthalmic solution was administered as ED (50 μL), a subconjunctival injection (SCI; 100 μL), or an IC injection (100 μL). The pharmacokinetics of LVFX were then investigated. The eyes ($n = 5$ per group) were enucleated 0 or 2 h after the lensectomy. The aqueous humor was collected with a 23-gauge needle. The eyeball was rinsed in sterile saline, immediately dipped in liquid nitrogen, and frozen. The regions from the corneal limbus to the posterior segment of the frozen eyes were dissected and separated into 3 equal parts using a razor. After dissection, only the vitreous humor was collected to avoid contamination with other tissues, weighed, and stored at −80°C. The concentrations of LVFX in the aqueous and vitreous humors were determined by high-performance liquid chromatography (HPLC). Briefly, for the assay of LVFX, internal standard (lomefloxacin) and 0.2% acetic acid solution were added to the sample, and the mixture was extracted by solid-phase extraction (Oasis MAX; Waters Corporation, Milford, MA). The extract was injected into an HPLC system equipped with an analytical column (ACQUITY UPLC BEH Phenyl 1.7 μm, 100×2.1 mm i.d.; Waters Corporation) and the fluorescence intensity of LVFX was detected. The concentrations in the aqueous humor (μg/mL) and vitreous humor (ng/g) were calculated using a calibration curve.

Prevention of experimental E. faecalis*-induced endophthalmitis*

After lensectomy with intentional rupture of the posterior capsule, 0.1 mL of OG1S strain was inoculated into the anterior chamber using a blunt needle on a 1-mL tuberculin syringe. The rabbits were then divided into 4 groups and treated with 0.5% LVFX ED, 0.5% MFLX ED, IC LVFX, or IC saline. In the LVFX or MFLX ED group, 0.5% LVFX or 0.5% MFLX ED (50 μL) was administered 0, 3, and 6 h after surgery. The IC injection of 0.5% LVFX or saline (100 μL) was performed at the end of surgery in the IC LVFX and saline groups, respectively. The course of infection in the eyes of each group was monitored using clinical scores, electroretinography (ERG), and the quantification of bacteria recovered from samples collected as described previously.[16] Animals in which the study drug was not administered correctly or a sample was not collected accurately were excluded from the analysis.

Statistical analysis

Differences between the 2 groups were analyzed using Student's *t*-test. The Tukey–Kramer test was used for multiple comparisons. A P value of < 0.05 was considered to indicate statistical significance.

Results

Intraocular penetration of LVFX

The concentrations of LVFX 2 h after the application of ED or SCI were measured and compared. In the aqueous humor of eyes treated with ED, the concentration of LVFX in the eyes without capsule rupture was higher than in the eyes with capsule rupture, although the difference was not statistically significant ($P=0.18$; Fig. 1). LVFX in the SCI group could similarly penetrate to the aqueous humor in eyes with or without capsule rupture ($P=0.87$; Fig. 1). The LVFX concentration in the ED group was significantly higher in the aqueous humor in eyes without capsule rupture than in the SCI group ($P<0.05$; Fig. 1). The LVFX concentration in the ED group, but not in the SCI group, could exceed the MIC against OG1S. In each part of the vitreous body, the LVFX concentration in the SCI group was significantly higher in eyes with or without PCR than in the ED group, except in the posterior vitreous (Fig. 1). No difference in LVFX concentration was found in eyes with and without rupture in the ED or SCI group (Fig. 1). However, the LVFX concentration in neither the ED nor the SCI group reached the MIC against OG1S in the vitreous.

Next, we examined LVFX penetration in eyes treated with an IC injection of LVFX. In the aqueous humor, the LVFX concentration in eyes with or without capsule rupture was decreased from 0 to 2 h ($P<0.05$; Fig. 2). In each part of the vitreous, the LVFX concentration in eyes with rupture was higher than in eyes without rupture, although the differences were not statistically significant. The vitreous sustained high LVFX concentrations that greatly exceeded the MIC of OG1S for 2 h.

Prevention of endophthalmitis

The eyes were examined at the designated times to assess their intraocular inflammation scores (Fig. 3). The mean scores in the IC LVFX group were significantly lower than those in the IC saline group at 12, 24, and 48 h (12 h: $P<0.05$; 24 and 48 h: $P<0.001$). There were significant differences in the intraocular inflammation scores between the IC LVFX group and ED group (LVFX and MFLX) at 48 h ($P<0.001$). The intraocular inflammation score in the MFLX ED group at 24 h was also significantly lower than that in the IC saline group ($P<0.001$).

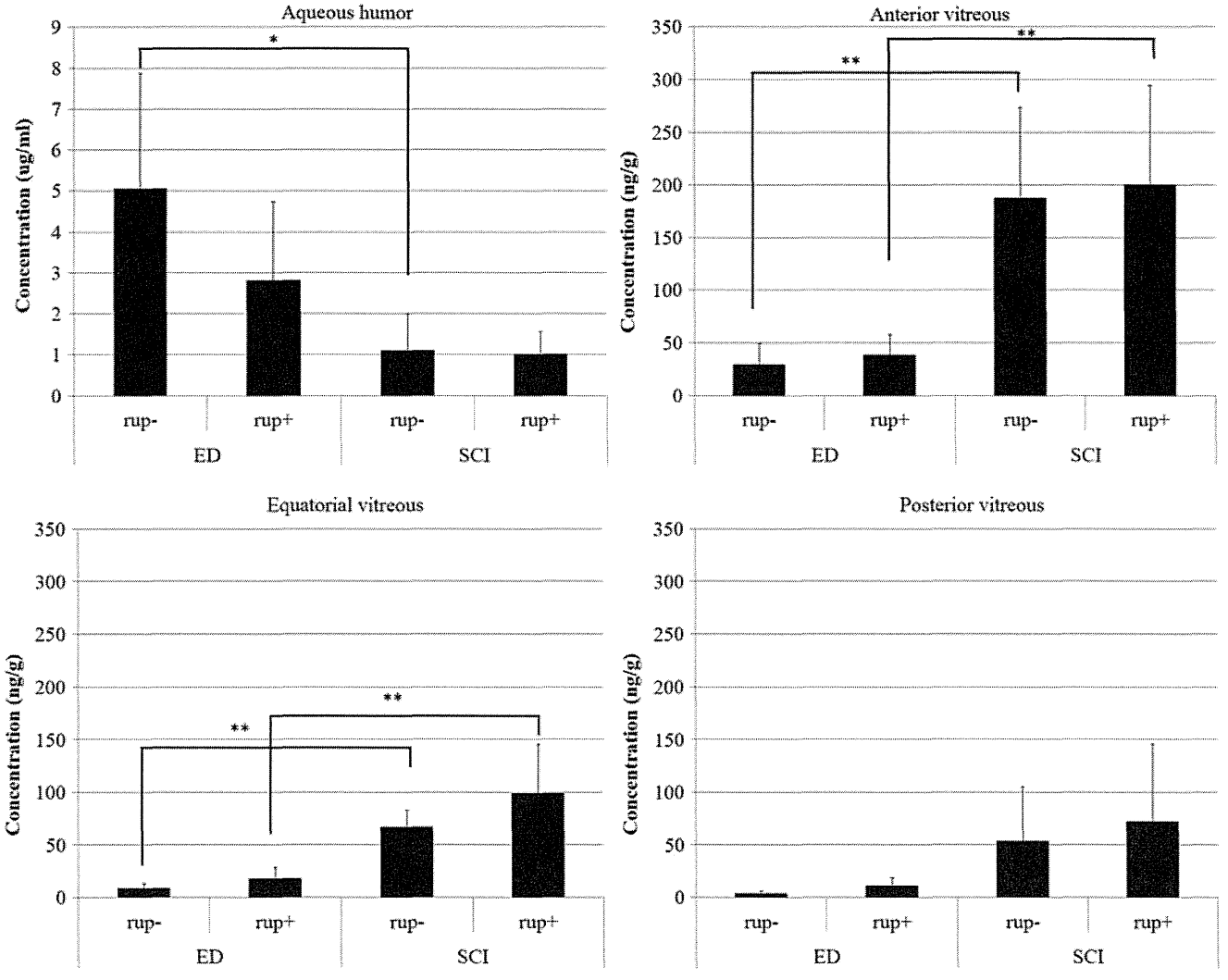

FIG. 1. Concentrations of LVFX in the aqueous and trisected vitreous (mean ± standard deviation, $n=5$) at 2 h after a single instillation of ED or SCI. $*P<0.05$ $**P<0.01$. ED, eye drop; SCI, subconjunctival injection; rup, rupture. −, minus; +, positive.

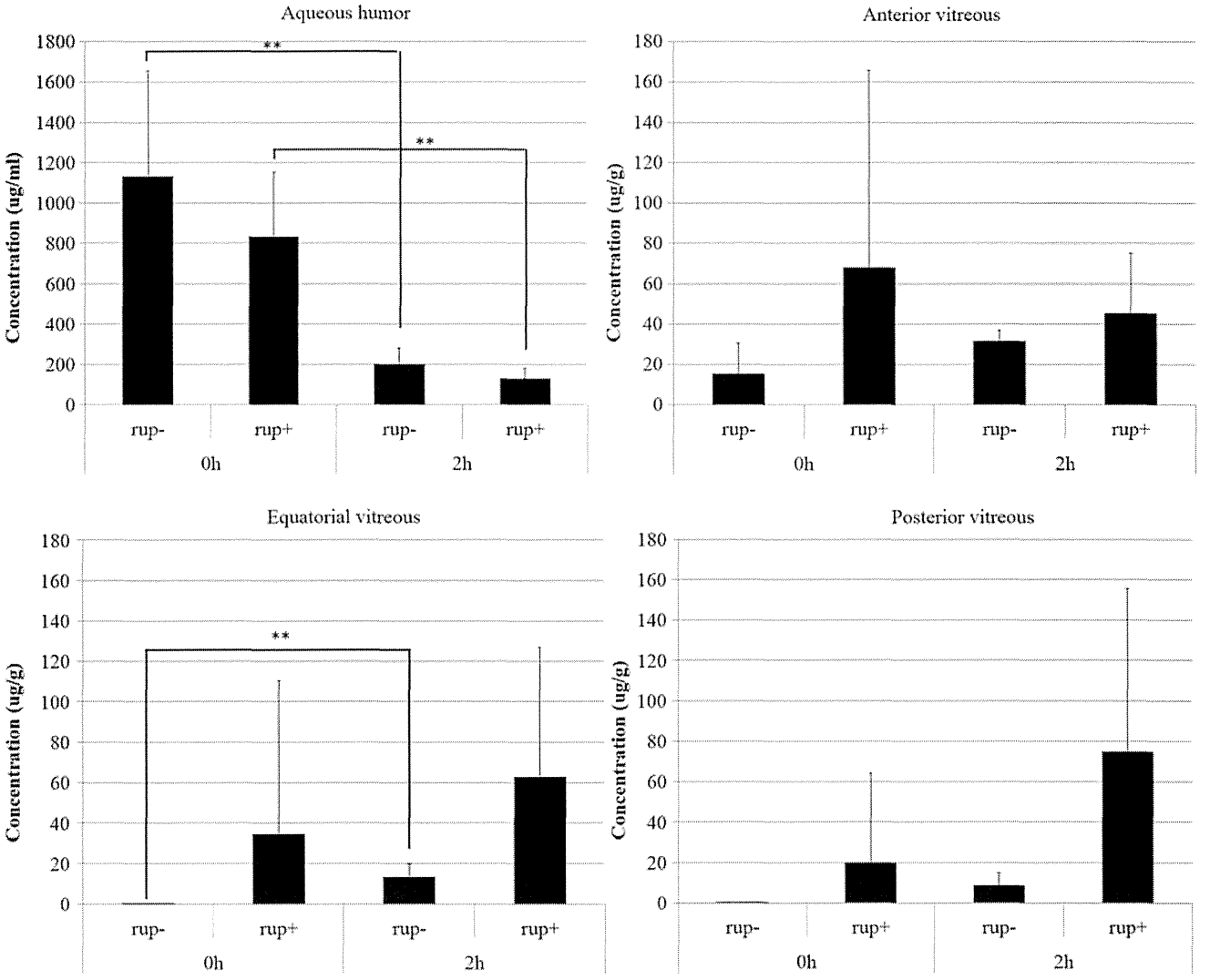

FIG. 2. Concentrations of LVFX in the aqueous and trisected vitreous (mean ± standard deviation, $n = 5$) at 0 and 2 h after a single IC injection. $^{**}P < 0.01$. IC, intracameral. −, minus; +, positive.

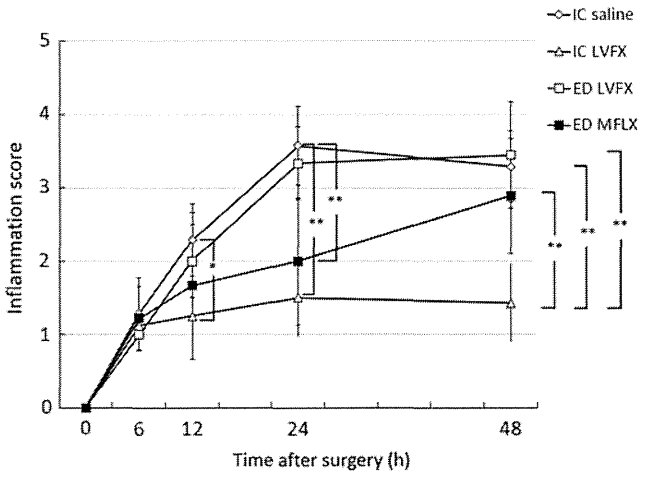

FIG. 3. Intraocular inflammation scores at the specified times after surgery and the induction of *Enterococcus faecalis* endophthalmitis. The data are the means ± standard deviations ($n = 7$–9). $^{*}P < 0.05$; $^{**}P < 0.001$. MLFX, moxifloxacin; LVFX, levofloxacin.

The retinal responsiveness of eyes infected with *E. faecalis* was determined by ERG (Fig. 4). Retinal function from 24 to 48 h was significantly greater in the IC LVFX group than in the IC saline (24 h: $P < 0.05$; 48 h: $P < 0.001$), LVFX ED (24 h: $P < 0.01$; 48 h: $P < 0.001$), and MFLX ED (48 h: $P < 0.001$) groups, except in comparison to the MFLX ED group at 24 h. There was no significant difference in retinal responsiveness between the IC saline and ED groups (LVFX and MFLX) from 24 to 48 h.

There was no significant difference in viable bacteria recovered from the anterior chamber among the 4 groups. In the vitreous, bacterial growth in the IC LVFX group (5.1 ± 1.1 $\log_{10}$ CFU/mL) was significantly reduced compared with that in the IC saline (8.3 ± 0.5 $\log_{10}$ CFU/mL; $P < 0.001$), LVFX ED (8.7 ± 0.9 $\log_{10}$ CFU/mL; $P < 0.001$), and MFLX ED (8.9 ± 0.7 $\log_{10}$ CFU/mL; $P < 0.001$) groups (Fig. 5).

Discussion

Postoperative endophthalmitis is a severe complication of cataract surgery; however, it may be effectively prevented using antibiotics. Immediate postoperative antibiotic administration is essential to improve bacterial killing and

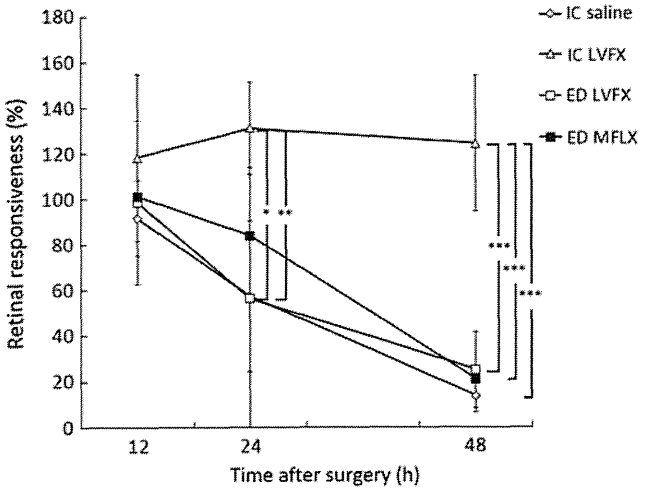

FIG. 4. Effects of antibiotics on electroretinographic measurements of retinal responsiveness at the specified times after surgery and the induction of *E. faecalis* endophthalmitis. The data are the means ± standard deviations ($n = 7–9$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

prevent infection by blocking the rapid penetration of pathogens from the anterior chamber to the vitreous. Rupture of the posterior capsule can induce bacterial translocation from the anterior chamber to the vitreous. Some investigators have reported that cataract surgery without PCR does not allow substances in the aqueous humor to move into the vitreous humor because the posterior capsule[17–19] and anterior vitreous membrane[20] form a firm barrier. Thus, ocular penetration by postoperative antibiotics could differ between eyes with and without PCR. Because fluoroquinolone EDs have broad spectra of action and excellent ocular penetration, they are widely used for the postoperative prophylaxis of endophthalmitis.[21] In our study, ocular penetration with different drug-delivery methods was compared using a 0.5% LVFX ophthalmic solution, which is widely used for surgical prophylaxis in Japan. This ophthalmic solution does not contain preservatives (e.g., boric acid and benzalkonium chloride) and the IC injection of LVFX was nontoxic in terms of the clinical toxicity score, corneal thickness, and cell viability.[22] Although it is difficult to exactly compare drug penetration between ED and SCIs because of differences in the amount of drug administered (ED, 50 μL; SCI 100 μL), LVFX ED can penetrate the aqueous humor more efficiently than SCI. In contrast, the LVFX concentration following SCI in the vitreous was higher than that following ED delivery. This confirms previous data that show that a potential barrier to ED is diffusion through the cornea, and subconjunctival routes could use the permeability of the sclera to penetrate the posterior segment.[23–28] Although not statistically significant, the LVFX concentration following ED delivery in the aqueous humor of eyes with PCR was lower than that in eyes without rupture. It is likely that the drug in the aqueous humor of eyes with PCR diffused to the posterior segment and therefore did not maintain a high concentration in the aqueous humor. In contrast, drug penetration with SCI would not be expected to be influenced by PCR because the drug could permeate through the sclera. The concentration of LVFX with ED or SCI at 2 h after administration was much lower than the MIC of *E. faecalis* (2 μg/mL). Because the 0.5% MFLX ophthalmic solution could penetrate the anterior chamber better than other fluoroquinolones,[29–31] it may penetrate the vitreous in eyes with rupture. In our study, the IC injection of LVFX resulted in a high concentration of LVFX in the aqueous humor and vitreous of eyes with or without PCR. Although not statistically significant, LVFX could better penetrate the vitreous of eyes with rupture versus those without rupture following IC injection. This indicates that IC injection could be effective at inhibiting the proliferation of bacteria that translocate through the PCR to the vitreous cavity.

Previous studies have shown the efficacy of antibiotics for preventing endophthalmitis in animal models. Kowalski *et al.*[32] demonstrated that ofloxacin and MFLX were more effective at preventing endophthalmitis than non-fluoroquinolone antibacterial agents in phakic rabbit eyes in which the anterior chambers were inoculated with *Staphylococcus aureus*. The SCI of a combination of triamcinolone and ciprofloxacin hydrochloride was useful for preventing endophthalmitis caused by *S. aureus* in phakic rabbit eyes.[33] Moreover, IC MFLX was effective in preventing endophthalmitis in a phakic rabbit model after an *S. aureus* intravitreal challenge.[34] However, these animal models may

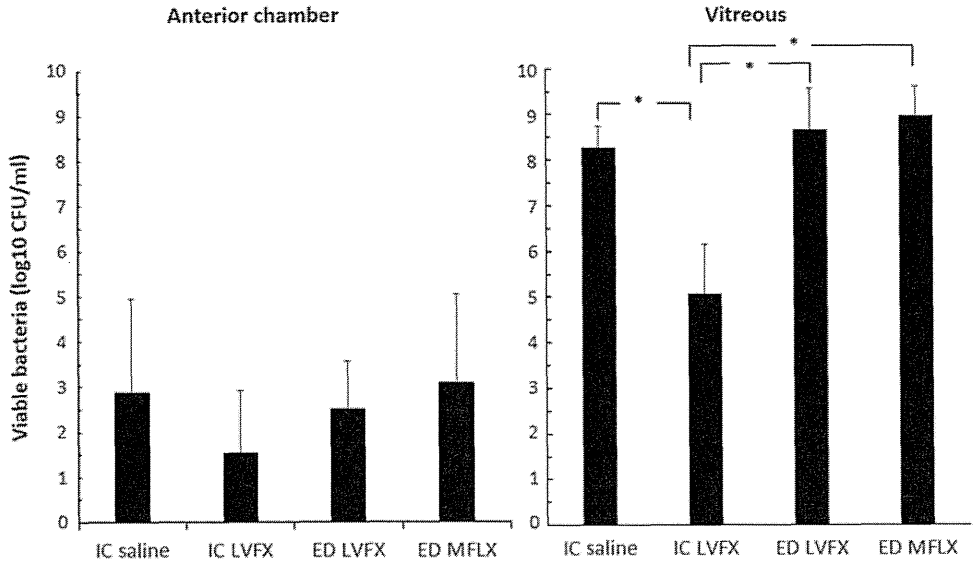

FIG. 5. Effects of antibiotics on bacterial counts in the anterior chamber (*left*) and vitreous (*right*). The data are the means ± standard deviations ($n = 5–9$). $^{*}P < 0.001$.

differ from actual postoperative endophthalmitis, where there is an anatomical barrier between the anterior chamber and vitreous cavity. Our model was established by inoculating pathogens into the anterior chamber with PCR after lensectomy, similar to an actual endophthalmitis case, especially those occurring after surgical complications (e.g., rupture). In this study, *E. faecalis* was used to create a model of endophthalmitis. Although *E. faecalis* is rarely a causative agent of clinical endophthalmitis, it can rapidly induce postoperative endophthalmitis, often within 2–4 days, and can cause substantial vision loss upon infection.[35–37] Indeed, patients with *E. faecalis*-related endophthalmitis had the worst visual outcome in the Endophthalmitis Vitrectomy Study.[38]

We found that the IC injection of LVFX significantly reduced inflammation scores and bacterial counts in the vitreous humor and maintained retinal function in our aphakic rabbit model, compared with LVFX ED, MFLX ED, and IC saline treatment. These results indicate that the IC injection of antibiotics could be effective for preventing endophthalmitis in cataract surgery with PCR. We used *E. faecalis*, for which the MICs of MFLX and LVFX are 0.5 and 2.0 μg/mL, respectively. The MICs of MFLX against ocular isolates, including staphylococci and streptococci, were lower than those of LVFX and gatifloxacin.[39,40] Thus, MFLX ED could be more effective in preventing endophthalmitis. Indeed, MFLX ED reduced the inflammation scores significantly at 24 h compared with IC saline. Because EDs were not administered from 6 h after surgery in this study, EDs could not inhibit bacterial growth in the anterior chamber or vitreous at 24 or 48 h. However, even EDs using MFLX could be less effective in preventing endophthalmitis in eyes with PCR because of reduced penetration to the vitreous and the existence of drug-resistant bacteria. We did not check the effect of IC MFLX for preventing endophthalmitis because we would like to reduce numbers of animal for experiment. Since IC MFLX can penetrate not only to the aqueous humor but also the vitreous in aphakic rabbit eyes,[41] it could be effective in preventing experimental endophthalmitis in complex phacoemulsification surgery. Moreover Matsuura *et al.* demonstrated that IC MFLX administration in clinical study decreased the risk for endophthalmitis by 3-fold. Thus, IC MFLX could prevent endophthalmitis in our model as well as IC LVFX.[42]

The results of our study should be interpreted with care, considering its limitations. First, this study was conducted in a rabbit model with experimentally induced *E. faecalis* endophthalmitis. Although this closely resembles the clinical situation, it is not identical to it. Second, the formulations of MFLX and LVFX used *in vivo* were the same as those used in clinical practice. Thus, the effective intraocular concentrations in this animal model may exceed those achieved in humans, considering the differences in eye size.

In conclusion, the results of this study indicate that the IC injection of antibiotics is effective in preventing endophthalmitis in complex phacoemulsification surgery compared with antibiotic ED. Prospective clinical studies are needed to confirm the potency, efficacy, and safety of IC injections for the prevention and treatment of bacterial endophthalmitis.

Acknowledgments

This study was supported, in part, by a Grant-in-Aid for scientific research (KAKENHI) from the Japan Society for the Promotion of Science (KAKENHI: Grants-in-Aid for Young Scientists B, 24791858). Technical expertise for measurement of drug concentrations was supported by Santen Pharmaceutical Co., Ltd.

Author Disclosure Statement

The authors have no commercial or financial interests associated with this article.

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Received: October 15, 2013
Accepted: November 13, 2013

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64064

Original Paper

Ophthalmic Research

Ophthalmic Res 2014;51:161–166
DOI: 10.1159/000357105

Received: June 26, 2013
Accepted after revision: October 31, 2013
Published online: March 5, 2014

Evaluation of Allergic Conjunctivitis by Thermography

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Key Words

Allergic conjunctivitis · Thermography · Allergen challenge test · Anti-allergic eye drops

Abstract

Purpose: To evaluate ocular surface temperature in assessing the efficacy of anti-allergic eye drops. ***Methods:*** Thirteen asymptomatic patients (24.7 ± 2.8 years) with proven seasonal allergic conjunctivitis due to cedar pollen were studied. A 0.025% levocabastine ophthalmic suspension was instilled in one eye (levocabastine eye) and artificial tears in the other eye (artificial tear eye) in a masked fashion 10 min prior to a conjunctival allergen challenge (CAC). Then, a drop of cedar pollen solution was dropped into the conjunctival sac to induce the allergic reaction. The surface temperature of the inferior bulbar conjunctiva was measured before and 30 min after the CAC with a newly developed non-contact ocular surface thermographer (OST). The degree of conjunctival injection and chemosis was also determined by slit-lamp biomicroscopy. The changes in the symptoms were evaluated by a questionnaire. ***Results:*** After the CAC, the temperature increased by 0.67 ± 0.10°C in the artificial tear eyes but by only 0.21 ± 0.06°C in the levocabastine eyes ($p < 0.05$). The score for conjunctival injection was 1.38 ± 0.24 and the chemosis score was 0.85 ± 0.25 for the artificial tear eyes and 0.62 ± 0.27 and 0.08 ± 0.08 in the levocabastine eyes ($p < 0.01$). The temperature increase was significantly correlated with the conjunctival injection scores ($r = 0.63$; $p < 0.001$). ***Conclusion:*** The significant correlation of the conjunctival surface temperature with the severity of the conjunctival allergic reaction indicates that the temperature measured by the OST can be used to objectively evaluate the efficacy of topical anti-allergic agents.

Introduction

Allergic conjunctivitis is one of the most common diseases seen by allergists and ophthalmologists. The hallmark symptoms and signs of allergic conjunctivitis are ocular itching, conjunctival redness, ocular chemosis, mucous discharge, feeling of heat and eyelid swelling [1]. Because of the increase in the incidence of allergic diseases, many clinical trials have been conducted worldwide on allergic conjunctivitis. However, the findings from these clinical trials on allergic conjunctivitis are inconclusive because of improper trial design and lack of objective validations [2].

Grading scores have been used to evaluate the conjunctival redness and oedema. Differences in the grading scores as well as in the evaluation of the effectiveness of therapeutic drugs often arise due to interobserver variations [3].

Another method to objectively assess allergic conjunctivitis is thermography. Thermography is a non-invasive

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imaging technique that uses infrared radiation reflected from an object to determine the temperature of the object. It has been used as an objective diagnostic tool in many medical fields such as blood circulation disorders, inflammatory diseases and oncology [3–5]. In addition, thermography is being used to detect the changes of skin temperatures, e.g. the small increases in temperature associated with the skin prick test, areas of atopic dermatitis and facial temperature in oral food challenge tests [6–8]. The nasal temperature has also been measured in cases of suspected atopic bronchial asthma after the basal provocation test [9]. Ocular surface temperature has also been investigated in several fields of application [10–12]. We have developed an instrument named the ocular surface thermographer (OST) which can measure the surface temperature of the eye non-invasively in dry eye patients [13].

Because the conjunctival surface temperature increases with a dilation of the capillary vessels in the conjunctiva [14], we hypothesized that the conjunctival hyperaemia in eyes with allergic conjunctivitis will lead to increases in the conjunctival surface temperature. To test this, we recorded the conjunctival temperature before and after inducing the allergic conjunctivitis, and examined the effectiveness of topical anti-allergic agents by thermography.

Methods

Subjects

Thirteen healthy, asymptomatic volunteers with clinically diagnosed seasonal allergic conjunctivitis due to cedar pollen were studied. This study was conducted in the summer and the fall to avoid the allergy season of cedar pollen. There were 8 men and 5 women with a mean ± standard deviation age of 24.7 ± 2.8 years and a range of 21–31 years. To be included, the subjects were required to have a history of clinically proven allergic conjunctivitis with a positive radio-allergosorbent test for allergic hypersensitivity to cedar pollen. All subjects had not used topical or systemic medications for allergic diseases for more than 3 months at the time of the tests. The purpose of this study and the procedures to be used were presented to all subjects, and a signed informed consent was obtained from each individual. This study protocol was approved by the Institutional Review Board of Ehime University and conformed to the tenets of the Declaration of Helsinki.

Medications

The conjunctival allergen challenge (CAC) test was performed to induce type 1 allergic conjunctivitis. The allergen solution used for the CAC test was the Allergen Scratch Extract Torii Cedar Pollen for Diagnostic Use (Torii, Tokyo, Japan). The allergen solution and control solution were diluted with phosphate-buffered saline by 100-fold. In preliminary experiments, all subjects were tested to determine whether the allergen solution diluted by 100-fold caused symptoms and signs of allergic conjunctivitis in both eyes [15–18].

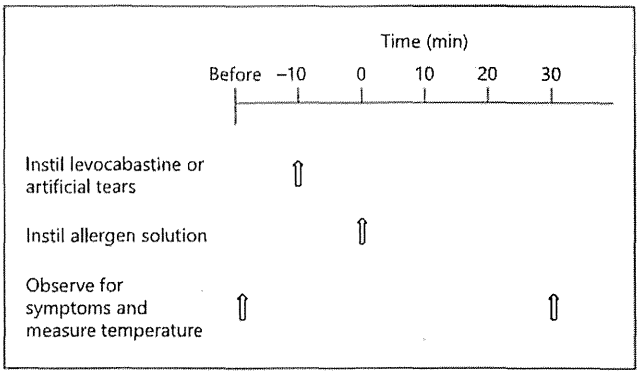

Fig. 1. Protocol of clinical study with the OST. Before beginning the experiment, all eyes were examined by slit-lamp biomicroscopy, and measurements of the lower conjunctival surface temperature were made. The temperature was measured with our OST. Then, one randomly selected eye received 0.025% levocabastine ophthalmic suspension, and the fellow eye received artificial tears. Ten minutes later, 50 µl of the CAC solution was instilled into both eyes. Thirty minutes later, the eyes were examined by slit-lamp biomicroscopy for signs of allergic conjunctivitis, and the conjunctival temperature was measured with the OST.

As expected, the instillation of the artificial tears and 0.025% levocabastine ophthalmic suspension without the allergen challenge did not lead to any signs or symptoms of allergic conjunctivitis.

To test the effect of an anti-allergy solution on the CAC-induced conjunctivitis, we used 0.025% levocabastine ophthalmic suspension on the experimental eyes and artificial tears for the control eyes.

Ocular Surface Thermographer

The newly developed OST (Tomey Corporation, Nagoya, Japan) is equipped with an infrared camera module (HX0830M1, NEC, Tokyo, Japan) and a colour CCD board camera (PKD-101, Pacific Co., Tokyo, Japan) [13]. The infrared radiation detector module is sensitive to infrared radiation between 8 and 12 µm, and radiation can be directed into either the infrared camera or the visible light camera. Thus, both infrared and visible light images can be recorded co-axially. An auto-alignment function is incorporated in the instrument to ensure that the instrument and object are maintained in a fixed location relative to each other. With this auto-alignment function, the position of the cameras with respect to the object to be measured can be held constant which allowed measurements of the ocular surface temperature to be performed at the same position.

Protocol

The protocol for this double-masked, placebo-controlled clinical study is shown in figure 1. Initially, all of the subjects underwent slit-lamp examination and measurements of the lower conjunctival surface temperature with the OST. These findings were used as the baseline values. If the subjects had any signs or symptoms of allergic conjunctivitis, they were excluded. Then, 1 drop of 0.025% levocabastine ophthalmic suspension was dropped onto

one (levocabastine eye) eye and the same amount of artificial tears onto the other eye (artificial tear eye). Ten minutes later, 50 µl of the allergen solution was dropped onto both eyes for the CAC. Thirty minutes after the CAC, the degree of conjunctivitis was determined by slit-lamp biomicroscopy, and the temperature was measured. The criteria for the scoring of slit-lamp findings and ocular symptoms are listed in table 1 [16].

The conjunctival temperature was measured with the OST in a standard clinical room maintained at a temperature of 26.5 ± 1.5°C and humidity of 42.5 ± 2.5% as reported [13]. The subjects were allowed to blink normally, and then they were asked to close both eyes for 5 s, and then open their eyes and look upward. The inferior bulbar conjunctival temperature was measured immediately after their eyes had been opened. The conjunctival vessels were used as markers for alignment, and the average conjunctival temperature was analysed. The inferior bulbar conjunctiva was defined as a circular region 4 mm in diameter with its centre at the midpoint of the pupil.

Statistical Analyses

All data are expressed as the means ± standard error of the means. Wilcoxon matched pairs signed-rank tests were used to compare the clinical scores after each allergen challenge test. Paired t tests were used to determine the significance of the differences of the conjunctival temperature before and after CAC in artificial tear and levocabastine eyes. Pearson's correlation coefficient was used for examining correlations. A p value <0.05 was considered statistically significant.

Table 1. Criteria used to determine the clinical score of the conjunctival surface

Redness	
0	None
1	Mild (dilation of a few blood vessels)
2	Moderate (dilation of many blood vessels)
3	Severe (dilation of all blood vessels, white of the eye is hardly distinguishable)
Chemosis	
0	None
1	Mild (slight oedema detectable only by slit-lamp)
2	Moderate (more diffuse oedema visible in normal room light)
3	Severe (ballooning of overall bulbar conjunctiva)
Itching	
0	None
1	Mild (intermittent itching)
2	Moderate (continuous itching)
3	Severe (continuous itching with the desire to rub, normal functioning not impaired)

The scores 0–3 for conjunctival redness and chemosis were determined by slit-lamp biomicroscopy, and the symptom of itching was evaluated by a questionnaire.

Results

Effect of Levocabastine on Signs and Symptoms Induced by CAC

Before the CAC, all subjects had no signs or symptoms of allergic conjunctivitis. After the CAC, the average redness, chemosis and itching scores in the levocabastine eyes were significantly lower than those in the artificial tear eyes ($p < 0.01$; Wilcoxon signed-rank test; table 2).

Table 2. Effect of levocabastine on clinical scores and symptoms after CAC

	Artificial tears	Levocabastine
Redness	1.38±0.87*	0.62±0.96*
Chemosis	0.85±0.90*	0.08±0.28*
Itching	1.62±0.67*	0.08±0.28*

* $p < 0.01$: statistically significant. The redness and chemosis of the bulbar conjunctiva were significantly lower in levocabastine than artificial tear eyes ($p < 0.01$; Wilcoxon signed-rank test). The itching score was also significantly lower in levocabastine than artificial tear eyes ($p < 0.01$; Wilcoxon signed-rank test).

Effect of Levocabastine on Conjunctival Surface Temperature Induced by CAC

Typical thermographic and photographic images of the anterior surface of the eye before and after the CAC are shown in figure 2. The surface temperature of the inferior bulbar conjunctiva measured in the 4-mm circle before the CAC was 34.29 and 34.48°C. The mean surface temperature in all eyes before the CAC was 34.26 ± 0.18°C, and there was no difference between the right and left eyes.

Thermographic and photographic images after the CAC are shown in the lower half of figure 2. The surface temperature of the inferior bulbar conjunctiva in the circle was 34.92°C in the eye that had received the artificial tears, and it was 34.64°C in the eye instilled with levocabastine. The increase in the temperature after CAC was significantly greater in the artificial tear eyes (0.67 ± 0.10°C) than in the levocabastine eyes (0.21 ± 0.06°C; $p < 0.001$; paired t test; fig. 3).

Correlations between Conjunctival Surface Temperatures and Clinical Scores

The correlation coefficient was calculated between the change in the conjunctival surface temperature after the CAC and the sign and symptom scores (table 3). Statistical

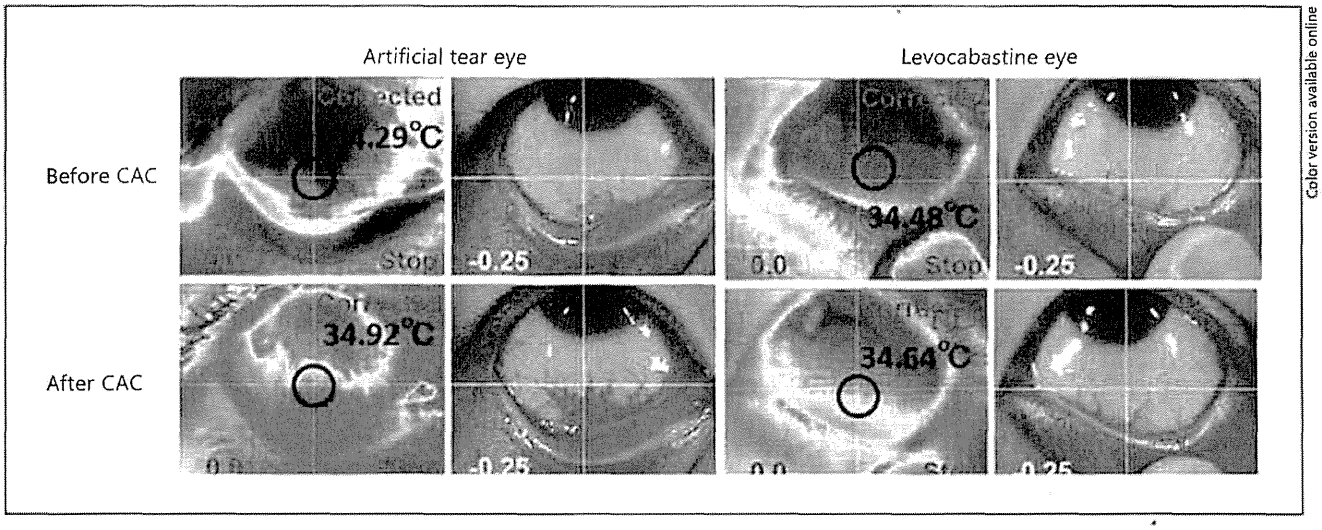

Fig. 2. Effect of levocabastine on conjunctival surface temperature induced by CAC solution. Representative thermographic and photographic images before (upper panels) and 30 min after the CAC (lower panels). The surface temperature of the inferior bulbar conjunctiva before the CAC was 34.29°C (upper left) and 34.48°C (upper right). The images of an eye instilled with artificial tears (bottom left) and an eye instilled with levocabastine (bottom right) after the CAC. The temperature after the CAC was 34.92°C in the artificial tear eye and 34.64°C in the levocabastine eye.

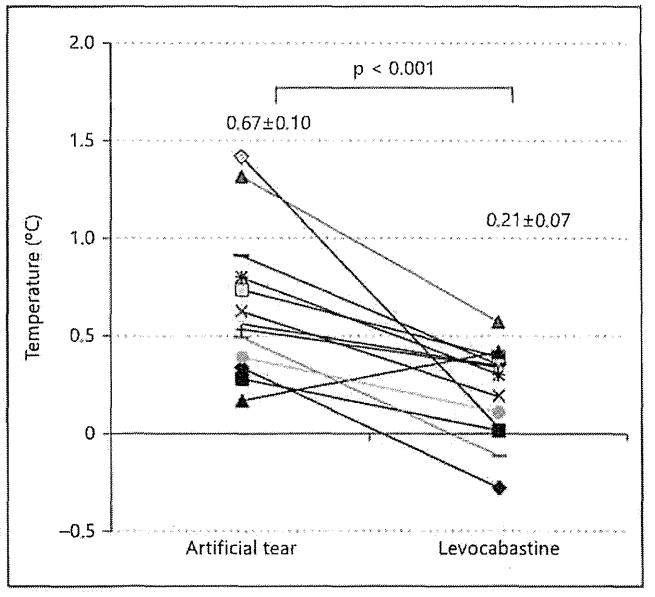

Fig. 3. Average changes in conjunctival temperature after the CAC in eyes pretreated with levocabastine or artificial tears. The increase in the temperature was significantly higher in the artificial tear eyes (0.67 ± 0.10°C) than in the levocabastine eyes (0.21 ± 0.06°C; $p < 0.001$; paired t tests).

Table 3. Correlation coefficients between conjunctival surface temperature and clinical scores

Clinical score	n	Change of temperature, °C	Correlation coefficient
Redness			
0	9	0.22±0.06	0.63 (p = 0.00058)
1	11	0.39±0.09	
2	3	0.77±0.33	
3	3	0.94±0.22	
Chemosis			
0	17	0.32±0.09	0.39 (p = 0.046)
1	7	0.63±0.15	
2	1	0.5	
3	1	0.92	
Itching			
0	13	0.34±0.11	0.38 (p = 0.056)
1	5	0.32±0.17	
2	7	0.63±0.13	
3	1	0.92	

Statistical analysis showed no significant correlation between the conjunctival temperature and chemosis and itching scores. The change of temperature was significantly correlated with the conjunctival redness.

analysis showed a significant correlation between the conjunctival temperature and the conjunctival redness ($r = 0.63$; $p = 0.00058$). However, the temperature change was not significantly correlated with the chemosis and itching scores.

Discussion

Our results showed that the conjunctival surface temperature increased significantly in patients with allergic conjunctivitis elicited by the CAC, and that levocabastine, an anti-allergic agent, was able to suppress the signs and symptoms of the conjunctivitis. The suppression of the signs and symptoms was accompanied by a reduction in the temperature rise after the CAC.

To the best of our knowledge, this is the first study that used thermography to determine the changes of conjunctival temperature during an allergic reaction induced by a CAC. CAC is a method of inducing allergic conjunctivitis with itching and increased hyperaemia and oedema [15–18]. In most clinical studies of allergic conjunctivitis using CAC, the main parameters measured for the degree of conjunctivitis were the symptoms and signs observed by slit-lamp biomicroscopy. These scores are dependent on the sensitivity of the subjects to the symptoms.

To overcome the inter- and intra-observer reliability, objective methods of measuring the bulbar redness have been used [19, 20]. Although these methods have been reliable with less variation, they require considerable time to analyse the changes.

Earlier, Fukushima and Tomita [21] and Takahashi et al. [22] reported that image analyses can be used to quantitatively evaluate the conjunctival hyperaemia and oedema in guinea pigs with histamine-induced hyperaemia or an allergic reaction. However, their methods cannot be easily used in humans because they require the injection of histamine.

Thermography is a non-invasive imaging technique which has been used for the diagnosis and evaluation of the therapeutic effect of drugs on other organs. Thus, Laino and Di Carlo [23] and others [24, 25] reported that thermography was an objective way to evaluate the patch test in allergic contact dermatitis where clinical assessment is difficult. Clark et al. [8] reported that thermography can be used to detect the early changes of the facial surface temperature when food allergy develops.

Thermography has also been used to evaluate the changes of temperature in various ocular surface diseases. We have demonstrated that the temperature of a functional bleb is lower than that of a non-functional bleb [11], and the temperature of the corneal surface in eyes with the dry eye syndrome is lower than that in normal eyes [13]. It has also been reported that thermography is useful in evaluating bacterial corneal ulcers or the effect of reconstruction of lagophthalmos [26, 27].

Thermography has also been used to evaluate the degree of conjunctival hyperaemia and blood flow. Efron et al. [14] used a hypertonic saline solution to induce conjunctival hyperaemia, and they measured the conjunctival temperature by an infrared bolometer. They showed that the degree of conjunctival hyperaemia was correlated with the change in the conjunctival temperature. Duench et al. [28] measured the conjunctival temperature together with conjunctival blood flow and redness throughout the day and showed that these three parameters were significantly correlated. Thus, an increase in ocular surface temperature has been observed when blood flow increases during inflammation of the anterior segment of the eyes. Thermography was able to detect the conjunctival temperature of allergic conjunctivitis which is supposed to be higher than that of the normal conjunctiva. However, Rimas et al. [29] did not detect significant changes of the ocular surface temperature by thermography, even though they also used CAC and measured the clinical signs and symptoms in their subjects. However, they stated that their thermographer might not be sensitive enough to detect the slight temperature changes. However, our OST was specially designed for the ocular surface and can measure the temperature more accurately and with greater sensitivity [13]. Furthermore, the ocular surface temperature measured by the OST has been reported to be highly reproducible [30]. The differences in the instruments may be the reason for the differences between the findings of Rimas et al. and our study.

We also demonstrated that levocabastine hydrochloride can reduce the changes induced by CAC. Levocabastine hydrochloride is an H_1 receptor antagonist, and it is effective in reducing the clinical scores of itching and redness after CAC [16]. In this study, the relationship of conjunctival temperature to chemosis and itching scores was not statistically significant probably because the conjunctival temperature reflected the filling of the vessels and not conjunctival oedema. Indeed, our results clearly showed that the conjunctival temperature was lower in the levocabastine than the artificial tear eyes, and also that the change of temperature was significantly correlated with the clinical scores of conjunctival redness. These results indicated that our OST is sensitive enough to detect the change of temperature during anti-allergic therapy, and that the OST can be used to evaluate the therapeutic effect of different agents against allergic conjunctivitis.

There are limitations to our experiment. Our findings showed that the individual conjunctival temperature varied among the subjects (fig. 3); thus, it would be difficult to diagnose allergic conjunctivitis by only the absolute value of the conjunctival temperature. Although the difference between conjunctival and body temperature could be examined, it would require further refinement of our OST to do this. However, the measurement of conjunctival temperature of the same subjects during the course of a disease process should be useful in determining the status of the disease and the effectiveness of the treatment.

In conclusion, the OST can detect changes of the conjunctival temperature with a high degree of reproducibility and sensitivity of conjunctival allergic reaction. These findings indicate that it can be useful in evaluating the efficacy of topical anti-allergic agents in cases of conjunctival allergies.

Disclosure Statement

There are no conflicts of interest and source of funding.

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J Infect Chemother 20 (2014) 407–411

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original article

Genotypic analysis of *Pseudomonas aeruginosa* isolated from ocular infection

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ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form
6 February 2014
Accepted 27 February 2014

Keywords:
Genotype
Pseudomonas aeruginosa
Keratitis
Conjunctivitis
Contact lens

ABSTRACT

Pseudomonas aeruginosa is the causative pathogen of keratitis, conjunctivitis, and dacryocystitis. However little is known about their clinical epidemiology in Japan. In this study we investigated the genotypic characterization and serotype of *P. aeruginosa* isolates from ocular infections. Thirty-four clinical *P. aeruginosa* isolates were characterized according to infection type, the type III secretion system (TTSS), serotype, and multilocus sequence typing (MLST). We divided the isolates into four clinical infection types as follows: Contact lens (CL)-related keratitis (CL-keratitis; 15 isolates), non CL-related keratitis (non CL-keratitis; 8 isolates), conjunctivitis (7 isolates), and dacryocystitis (4 isolates). Regarding the TTSS classification and serotyping classification, no significant differences were found among the infection types. Two clusters (I, II) and three subclusters (A, B, C) were classified according to MLST. CL-keratitis isolates with *exoU* positivity were clustered in II-B, and conjunctivitis was clustered in cluster I. Some linkage was found between the genetic background and CL-keratitis or conjunctivitis.

1. Introduction

Pseudomonas aeruginosa is a common opportunistic bacterial pathogen that causes various infections in humans, and it is a leading cause of keratitis worldwide. Keratitis caused by *P. aeruginosa* occurs following injury and ocular surgery or in association with contact lens (CL) wear and can progress rapidly with a suppurative infiltrate. Keratitis can lead to corneal perforation and melt, resulting in the loss of vision [1]. Along with keratitis, *P. aeruginosa* is a causative agent of conjunctivitis and dacryocystitis. Conjunctivitis caused by *P. aeruginosa* has been observed in neonatal intensive care unit infants or the elderly (age >70 years) [2,3]. Dacryocystitis caused by *P. aeruginosa* could form an abscess in the nasolacrimal sac that is frequently triggered by nasolacrimal duct obstruction [4]. To understand the mechanism of pathogenesis in ocular infections caused by *P. aeruginosa*, it is necessary to identify virulence factors, which are cell-associated structures such as flagella, pili, and lipopolysaccharide, as well as extracellular products, including proteases, exotoxin A and biofilm [5–11]. Along with these products, the type III secretion system (TTSS) is an important factor for pathogenicity in keratitis [12–16]. The TTSS transports toxins to host cells, and it includes a needle-like apparatus, effector protein, and pore-forming protein. Four effector proteins have been identified, namely ExoU, ExoS, ExoT, and ExoY. Among those proteins, ExoU and ExoS are considered the main factors of host cell cytotoxicity. The molecular structures of ExoS and ExoT show a striking likeness, and almost all *P. aeruginosa* strains have ExoT. Analysis of 63 isolates from ulcerative keratitis revealed that ExoU-positive strains predominate over ExoS-positive strains [17]. Moreover, Choy et al. demonstrated that strains with an *exoS+/exoU−* genotype predominated in non CL-related keratitis (non CL-keratitis) isolates, whereas the *exoS−/exoU +* genotype was associated with CL-related keratitis (CL-keratitis) isolates [18]. Thus, the TTSS could be related to pathogenicity in keratitis. Because tears contain antimicrobial components to protect the ocular surface, Gram-negative rods are rarely present in healthy conjunctival sacs. *P. aeruginosa* isolates need to survive in an ocular environment to cause infection, and some

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http://dx.doi.org/10.1016/j.jiac.2014.02.007
1341-321X/

strains with a specific TTSS type might adapt to the conditions of the ocular site. However, few studies have compared TTSS types among ocular infections such as keratitis, conjunctivitis, and dacryocystitis.

To determine the characteristics of *P. aeruginosa* strains isolated from ocular infections, it is critical to analyse their genotype. A combination of phenotypic and genotypic characteristics in a large survey showed that environmental and clinical *P. aeruginosa* isolates are indistinguishable [19,20]. By contrast, Stewart et al. revealed a genetic cluster in *P. aeruginosa* causing keratitis using the ArrayTube genotyping system [21]. Moreover, 71% of isolates from United Kingdom keratitis cases were clustered, and the *P. aeruginosa eccB* clonal complex is associated with adaptation to survival in environmental water [22]. However, similar results have not been reported in other geographic areas, and no study has investigated the differences in genotypes of *P. aeruginosa* isolated from CL-keratitis, non CL-keratitis, conjunctivitis, and dacryocystitis. Multilocus sequence typing (MLST) is widely used for genotyping of *P. aeruginosa* due to the large, readily accessible database [23,24]. This database can be used to elucidate evolutionary relationships among strains and identify ancestral genotypes, as well as predict patterns of divergence within groups of related genotypes. eBURST, an MLST program, can be used with multilocus data to define groups or clonal complexes of related isolates derived from a common ancestor, the patterns of descent linking them, and the ancestral genotype. We report here a genetic Bayes classification of isolates from ocular infections using housekeeping genes from MLST—Bayes classification is a development of the conditional probability calculation and is useful for analysis of genetic diversity [25]. In the present study, *P. aeruginosa* isolates from ocular infections were compared by TTSS typing, serotype, and MLST.

2. Materials and methods

2.1. Bacterial strains and DNA isolation

Thirty-four *P. aeruginosa* isolates from various types of ocular infection were obtained from the Ehime University Hospital collection from 2003 to 2011. Tested isolates included 15 CL-keratitis, 8 non CL-keratitis, 7 conjunctivitis, and 4 dacryocystitis isolates. DNA extraction was performed using a DNA mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

2.2. Polymerase chain reaction (PCR)

The TTSS effector genotype of each clinical isolate was determined using multiplex PCR to detect the *exoS*, *exoT*, *exoY*, and *exoU* genes in a single reaction as described and optimized by Ajayi et al. [26] The conditions of PCR amplification were as follows: 2 μl of 10 × PCR buffer, 1.6 μl of dNTPs, 0.4 μl of DMSO, 0.8 μl of primer mix (F + R mixture; 25 μM), and 0.1 μl of ExTaq. The primer sequences of *exoU* are F-5′-GGGAATACTTTCCGGGAAGTT-3′ and R-5′-CGATCTCGCTGCTAATGTGTT-3′ and those of *exoS* are F-5′-ATCGCTTCAGCAGAGTCCGTC-3′ and R-5′-CAGGCCA-GATCAAGGCCGCGC-3′.

2.3. Serotyping

The O serotypes of the isolates were determined using a slide agglutination test kit containing three polyvalent antisera and 14 monovalent antisera (Denka Seiken Co., Tokyo, Japan). We applied the standard classification of O types from A to N proposed by the Serotyping Committee for the Japan *P. aeruginosa* Society [27].

Table 1
Prevalence of ExoS/U according to infection type.

Disease (N)	TTSS type		
	ExoS (N/%)	ExoU (N/%)	Other (N/%)
CL-keratitis (15)	8 (53.3%)	7 (46.7%)	0 (0%)
Non CL-keratitis (8)	6 (75%)	1 (12.5%)	1 (12.5%) No exo
Conjunctivitis (7)	4 (57.1%)	3 (42.9%)	0 (0%)
Dacryocystitis (4)	3 (75%)	0 (0%)	1 (25%) ExoS/U
Total	21 (61.8%)	11 (32.3%)	2 (5.9%)

2.4. MLST and Bayes classification

The MLST technique was conducted according to the guidelines at http://pubmlst.org/paeruginosa/, which were developed by Keith Jolley, and new alleles were sent to curator Eleanor Pinnock [28]. The clustering of STs was analysed using the eBURST (electronic Based Upon Related Sequence Types) algorithm (http://www.eburst.mlst.net). Bayes classification using seven housekeeping alleles was performed as described previously [25].

2.5. Statistical analysis

Comparison of genotype prevalence among ocular infections was performed by chi-squared double classification with one degree of freedom. A *P* value less than 0.05 was deemed to indicate statistical significance.

3. Results

3.1. Distribution of TTSS genes and serotyping

In the TTSS classification, 21 isolates (61.7%) were PCR-positive for *exoS*, 11 isolates (32.3%) were PCR-positive for *exoU*, 1 isolate was PCR-positive for both *exoS* and *exoU*, and 1 isolate was PCR-negative for both *exoS* and *exoU* (Table 1). No significant difference was noted among disease type regarding TTSS classification, although the rate of *exoU*-positive strains in CL-keratitis was higher than in the other diseases. In the serotyping classification, nine isolates (26.4%) were type G, six isolates (17.6%) were type B, five isolates (14.7%) were type E and I, three isolates (8.8%) were type C, two isolates (5.9%) were type K, less than 5% of isolates were other serotypes, and 1 isolate was non-typable (Table 2). No significant differences in serotype were observed among the disease types.

3.2. MLST and Bayes classification

Thirty-four *P. aeruginosa* isolates were classified according to disease type, the TTSS, and serotyping using the Bayes method. All

Table 2
Serotype according to infection type.

	CL-keratitis (N)	Non CL-keratitis (N)	Conjunctivitis (N)	Dacryocystitis (N)	Total (N)
A		1			1
B	3	2		1	6
C	3				3
E	4	1			5
F			1		1
G	3	1	4	1	9
I		2	2	1	5
K		1		1	2
M	1				1
No type	1				1
Total	15	8	7	4	

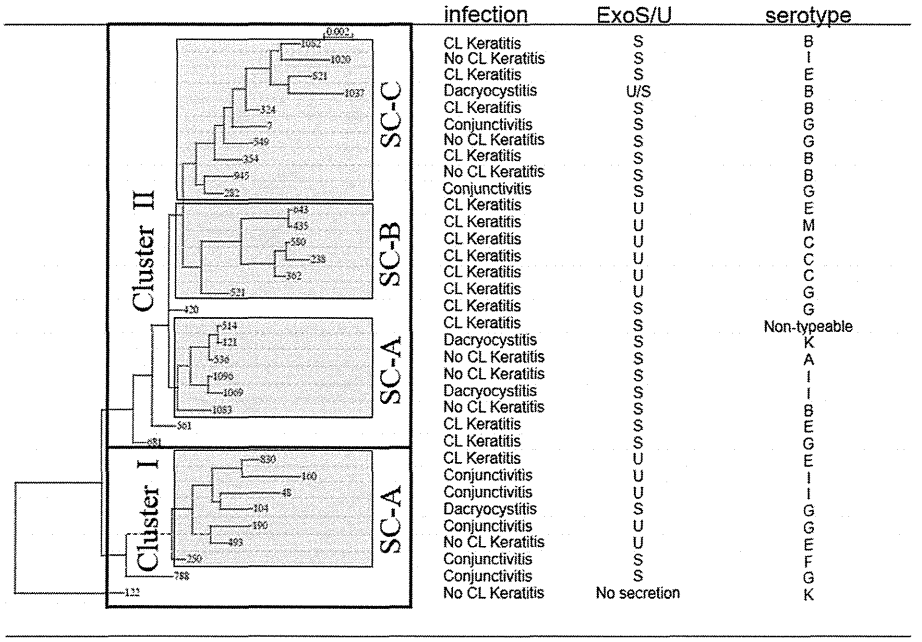

Fig. 1. Phylogenetic relationships among ocular isolates determined by MLST analysis. Tree showing the genetic relationships between the merged sequences of seven housekeeping gene fragments from *P. aeruginosa* isolates, analysed by Bayes classification. SC; subcluster.

base sequences of seven loci (housekeeping genes) were calculated using the Bayes method. Two clusters (I and II) and three subclusters (A, B, and C) were classified using the Bayes classification system (Fig. 1). *exoU*-positive strains were predominant in cluster I (5/8; 62.5%); by contrast, *exoS*-positive strains predominated in cluster II (18/25; 72.0%). Each subcluster had a specific TTSS type: e.g., I-A [*exoU*; 5/6 (83.3%)], II-A [*exoS*; 6/6 (100%)], II-B [*exoU*; 6/6 (100%)], and II-C [*exoS*; 9/9 (100%), one strain had both *exoS* and *exoU*]. Although II-B and II-C contained predominantly serotype C (3/6; 50%) and B (5/10; 50.0%), respectively, other subclusters did not have predominant serotypes. There were significant differences in the prevalence of CL-keratitis and conjunctivitis between clusters I and II. CL-keratitis belonged to cluster II significantly [cluster I: 1/9 (11.1%) vs. cluster II: 14/25 (56%); $P < 0.05$]. Conjunctivitis belonged to cluster I significantly [cluster I: 5/9 (55.6%) vs. cluster II: 2/25 (8.0%); $P < 0.01$] (Table 3). Notably, all isolates in II-B were CL-keratitis isolates, which harboured the *exoU* gene. Furthermore, conjunctivitis isolates and non CL-keratitis isolates were predominant in I-A (4/7; 57.1%) and II-A (3/6; 50%).

Alleles of the seven housekeeping gene in ocular isolates were investigated. The prevalence of *aroE* allele 5 in keratitis isolates was higher than in non CL-keratitis isolates [keratitis isolates: 82.6% (19/23); non-keratitis isolates: 27.3% (3/11)].

In the eBURST analysis, the genotype of ocular isolates was not clustered, except for several CL-keratitis isolates (Fig. 2).

4. Discussion

In the present study, *exoS*-positive strains were predominant in ocular isolates, including CL-keratitis, non CL-keratitis, conjunctivitis, and dacryocystitis isolates. In keratitis isolates, the *exoS*- and *exoU*-positive rates were 56.5% and 34.8%, respectively. However, the rate of *exoU*-positive strains in CL-keratitis was higher than that in other disease. Thus, *exoU* positivity could be associated with CL-keratitis isolates. These results are consistent with those in previous studies [18,22]. Moreover, CL-keratitis with *exoU* positivity was found to be clustered by MLST analysis. As Shanker et al. described, strains with certain genetic backgrounds might adapt to survive in environmental water [22]. Interestingly, non CL-keratitis with *exoS* positivity was not clustered in MLST classification. The origin of isolates could be different between CL-keratitis and non CL-keratitis. *P. aeruginosa* causing CL-keratitis might originate from environmental water, surviving various types of disinfectant systems, such as chlorine in tap water or CL disinfectant, including hydroperoxide or polyhexamethylene biguanide. Several other

Table 3
Relationship between cluster and infection type.

Cluster (N)	Subcluster (N)	Disease								TTSS type (N/%)[a]	
		CL-keratitis (N)		Non CL-keratitis (N)		Conjunctivitis (N)		Dacryocystitis (N)		S	U
I (8)	A (6)	1	1	1	1	3	5	1	1	0 (0%)	6 (100%)
	others (2)	0		0		2		0		2 (100%)	0 (0%)
II (25)	A (6)	1	14	3	5	0	2	2	3	6 (100%)	0 (0%)
	B (6)	6		0		0		0		0 (0%)	6 (100%)
	C (9)	4		1		2		1		9 (100%)	0 (0%)
	others (4)	3		1		0		0		4 (100%)	0 (0%)
P value[b]			$P < 0.05$		NS		$P < 0.01$		NS		

[a] Rate of the TTSS type in the subcluster.
[b] Prevalence of each infection type; cluster I vs. cluster II.

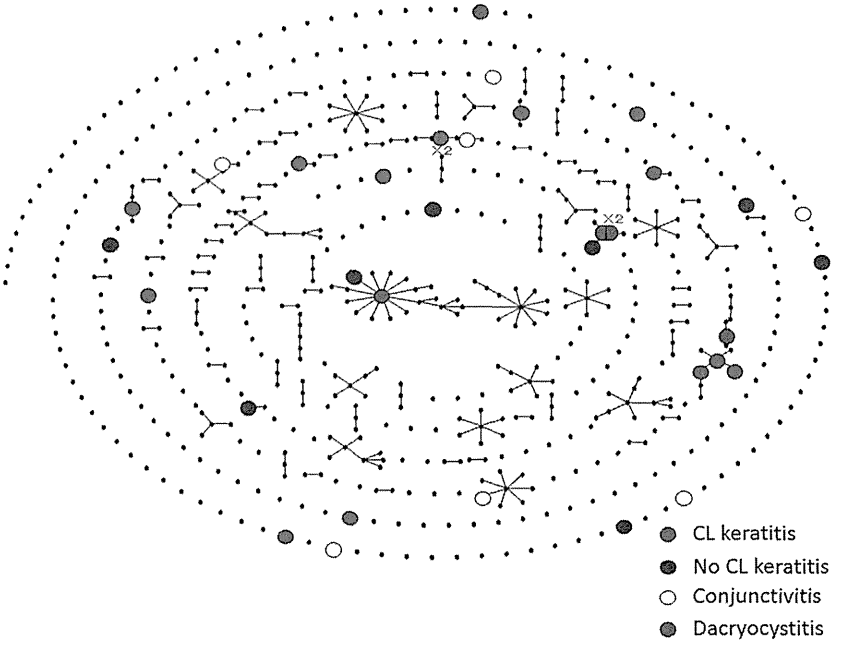

Fig. 2. eBURST analysis of P. aeruginosa isolates. Each dot represents an individual sequence type (ST). Two dots connected to a single line differ in only one locus. CL-keratitis (in red), non CL-keratitis (blue), conjunctivitis (yellow), and dacryocystitis (green) are represented.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

studies have also shown a relationship between exoU expression and resistance of *P. aeruginosa* to CL disinfectants [29], and to multiple antimicrobials [30]. Thus *P. aeruginosa* strains with *exoU* positivity could survive CL disinfectants and cause keratitis. In these processes, strains with a similar genetic background could be selected. Because these data in Japan were the same as in previous studies performed in the United Kingdom, similar results could be found in different geographical locations [21,22]. By contrast, non CL-keratitis is usually triggered by trauma, severe ocular surface disease, and dacryocystitis. Non CL-keratitis could originate from various sites, such as the environment, including not only environmental water but also soil, and the human microflora. Thus, CL-keratitis isolates are not clustered. In the present study, conjunctival isolates were clustered according to MLST analysis. *P. aeruginosa* is a rare pathogen of conjunctivitis and can cause this infection in infants, elderly people, and immunocompromised hosts. Thus, isolates with a similar genetic background might spread to patients in a hospital.

Our MLST data are similar to those in studies using the ArrayTube genotyping system [21,22]. However, the large cluster in the eBURST analysis, similar to that obtained with the ArrayTube genotyping system, was not found in our study. Several reasons should be considered. First, this study used a small population, including various types of ocular infectious disease, compared with previous studies. Thus, the genetic cluster could be small. Second, there are differences between the MLST and ArrayTube genotyping systems. MLST can scan only genetic informative traits of the core genome; by contrast, the ArrayTube genotyping system analyzes the composition of the accessory genome through a set of 38 genetic markers as well as the core genome. Further investigation is needed to determine the characteristics of *P. aeruginosa* ocular isolates.

In conclusion, CL-keratitis and conjunctivitis strains, but not the non CL-keratitis strain, were found to be clustered in the genetic analysis, suggesting that *P. aeruginosa* does not adapt to the conditions in the eye but can adapt to external environmental conditions.

Conflict of interest

S.Y. is employees of ROHTO Pharmaceutical Co., Ltd (Osaka, Japan). The remaining authors declare no conflict of interests.

Acknowledgements

We thank Hitoshi Miyamoto and Shinobu Murakami for their valuable technical assistance with our experiments and providing isolates. This study was supported by the Department of Bioscience, INCS, Ehime University.

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