IOP increases, the posterior chamber-anterior hyaloid membrane barrier will undergo an elevation of pressure [5]. We confirmed that the fluorescein beads could be trapped by the dense network of zonular fibers during standard PEA (Additional file 2, Normal), indicating that the zonular fibers may act as an important barrier for the invasion of bacteria from entering the vitreous cavity. Furthermore, our method also clearly documented the formation of a AHM tear [10], and the tear could be a risk factor leading to endophthalmitis following uneventful surgery (Additional file 2, AHT).

There are some limitations in this study. First, although a brief freezing of the outer surface of the porcine eye with liquid nitrogen was helpful for rapid and effective bisection of the eye, one could argue that this treatment might have affected the anatomical integrity of the PCSs. However, our thermographic measurements showed that the temperature around the zonular fibers after dipping the eye into liquid nitrogen for 5 seconds was around 4°C, and scanning electron microscopy showed that the morphology of the PCSs was normal (data not shown). Thus, we conclude that the morphology of the PCSs was preserved during this procedure.

The second limitation was that we were unable to complete the standard PEA in 1 out of 10 processed eyes because of the loss of the seal of the scleral rim to the glass slide. This warrants future studies on ways to obtain stronger sealing for this side-view imaging technique.

Conclusion

In conclusion, our side-view imaging technique can be a useful method of monitoring the movements of PCSs and the movement of surgical instrument during cataract surgery. It can be used for surgical training or evaluation of surgical procedures.

Additional files

Additional file 1: Side-viewing technique during phacoemulsification and aspiration. Side-view technique shows images of the PCSs and the movement of surgical instruments during PEA and insertion of the intraocular lens in an enucleated porcine eye. The zonular fibers, lens capsule, surgical instruments, and insertion of the intraocular lens can be seen. The right lower part of a screen is the surgeon's view.

Additional file 2: Observation of the flow of the irrigation fluid. The zonular fibers are stained by 1.0-μm fluorescein beads and the flow of the irrigation solution in the capsule can be seen. This shows an anterior hyaloid membrane tear (AHT).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YT, NM and XZ: Conception and design, acquisition, analysis and interpretation of data, drafting of manuscript, administrative and technical support. YT, TS, SK, XZ and AS: Technical support and analysis and interpretation of data. TU, KM, XZ and YO: Supervision. Read and approved the final manuscript. All authors approved the manuscript for submission.

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Yoshitaka Tasaka, and Noriyoshi Minami, contributed equally as co-first Authors.

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Utility of Real-time PCR Analysis for Appropriate Diagnosis for Keratitis

Tomoyuki Inoue, MD, and Yuichi Ohashi, MD

Abstract: Real-time polymerase chain reaction (PCR) is a quantitative method to measure the amount of amplified PCR product in real time with high sensitivity. We have applied this method to detect pathogens in cases of keratitis with an unknown cause. The scraped corneal epithelium for epithelial keratitis or aqueous humor for stromal or endothelial keratitis was obtained and DNA was extracted. The DNA from specific pathogens was amplified using specific primers and TaqMan probe, and assessed quantitatively. Here, we review previously reported noteworthy examples of keratitis diagnosed by our real-time PCR system as follows: cases with *Acanthamoeba* keratitis whose causative pathogen was only detected by real-time PCR despite not being detected by histological examination and culture; zoster sine herpete with atypical pseudodendrite; acyclovir-resistant herpetic keratitis estimated by changes in viral DNA copy numbers before and after treatment; and corneal endotheliitis positive for cytomegalovirus, human herpes virus-7, or human herpes virus-8. Real-time PCR helps ophthalmologists to make an early diagnosis and provide appropriate treatment for keratitis with complex clinical appearances.

Key Words: real-time PCR, diagnosis, keratitis, herpes virus, *Acanthamoeba*

(*Cornea* 2013;32(Suppl):S71–S76)

Polymerase chain reaction (PCR) is a molecular technique that generates millions of copies of a particular DNA sequence from a few copies of template DNA. Real-time PCR is a quantitative method to measure the amount of amplified PCR product in real time with high sensitivity, and is now an indispensable technique used in the medical field for a variety of applications including the detection and diagnosis of infectious diseases. We have used this method to detect pathogens in patients with keratitis of an unknown origin including epithelial, stromal, or endothelial keratitis.[1–6] First, the scraped corneal epithelium for epithelial keratitis or aqueous humor for stromal or endothelial keratitis was obtained. Scraped corneal epithelium was obtained as described previously,[7] and an aliquot of 0.1 mL of aqueous humor was obtained using a 30-G needle from the affected eye.[8] DNA was extracted from samples, and real-time PCR was used to detect pathogen-specific DNA. The primers and probes of *Acanthamoeba* and human herpes virus (HHV) were described previously.[9–16] HHV includes all 8 types, herpes simplex virus type 1 (HSV-1) and type 2, varicella-zoster virus (VZV), Epstein–Barr virus, cytomegalovirus (CMV), and HHV-6, HHV-7, and HHV-8. The DNA fragments were amplified under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The clinical features were investigated by slit-lamp examination and compared with viral copy numbers monitored by quantitative PCR through the clinical course. In cases of keratitis with an unknown cause, various pathogens were detected in each type of keratitis. Here, we present noteworthy examples of keratitis that were diagnosed only by our real-time PCR system.

ACANTHAMOEBA KERATITIS WAS DETECTED BY REAL-TIME PCR BUT NOT BY HISTOLOGICAL EXAMINATION OR CULTURE

Case

A 27-year-old man, who had worn frequent replacement soft contact lenses, reported having eye pain and decreased vision for a week in his right eye.[1] His visual acuity was 20/2000, and a slit-lamp examination showed that he had keratitis with disciform lesions (Figs. 1A, B). The anterior chamber was inflamed. The right eye was normal, and the corneal sensitivity was normal in both eyes. Corneal scraping was performed for cytopathological examination, culturing, and PCR. The findings of cytopathological examinations including those of fluorescent microscopic examination with Fungiflora Y staining were negative, and the cultures for bacteria, fungus, or *Acanthamoeba* failed to grow any pathogens. However, *Acanthamoeba* DNA was detected by real-time PCR, which allowed us to make a diagnosis of *Acanthamoeba* keratitis. Based on the positive PCR result, the patient was treated for the amoeba using topical 0.05% chlorhexidine digluconate hourly, 1% natamycin ointment 4 times a day, and 0.5% levofloxacin eye drops 4 times a day, in addition to 200 mg of oral itraconazole. The subepithelial infiltration and anterior uveitis gradually improved (Figs. 1C, D). *Acanthamoeba* DNA could not be detected by real-time PCR during the recovery stage, and the best-corrected visual acuity (BCVA) returned to 20/16.

There was a discrepancy in the results for the existence for *Acanthamoeba* between real-time PCR and histopathological examinations, even with Fungiflora Y staining, which has been reported to stain *Acanthamoeba* cysts fluorescently for easy identification.[17] This discrepancy may have occurred

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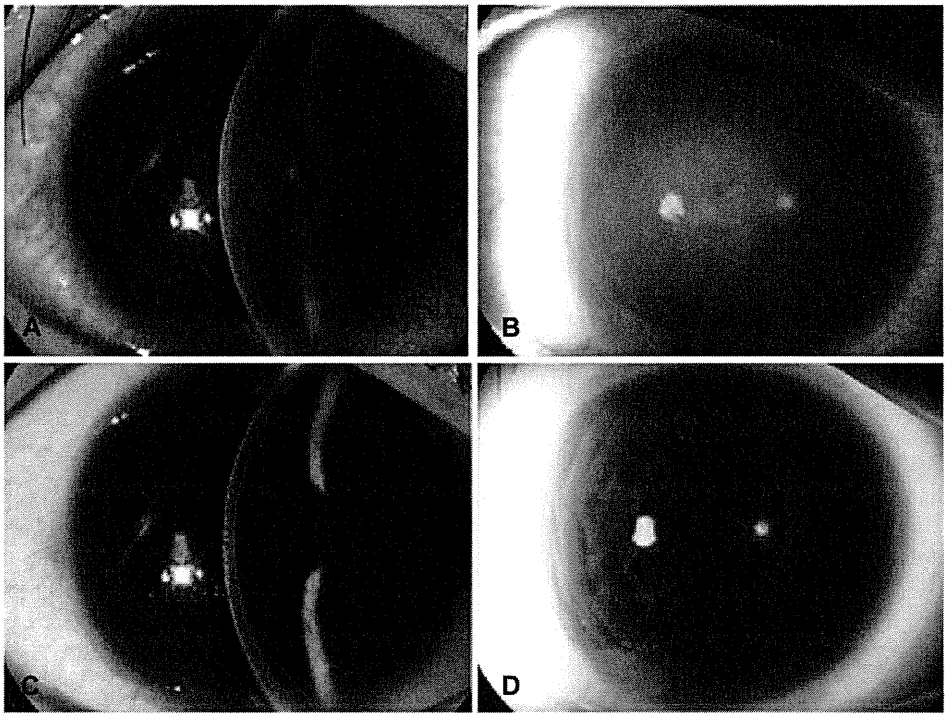

FIGURE 1. Slit-lamp photographs before (A, B) and 3 months after antiamoebic treatment (C, D). A and B, Keratitis with a disciform lesion and anterior chamber inflammation was observed. The visual acuity was 20/2000. C and D, Subepithelial infiltration and anterior uveitis were improved. The visual acuity returned to 20/16. Reprinted from Kandori et al[1] with permission from Wolters Kluwer Health.

because *Acanthamoeba* keratitis was diagnosed at an early stage when only trophozoites are present and the cysts may not be present. Despite there being a discrepancy, our case responded well to the antiamoebic drugs selected on the basis of our PCR results. The improvements validated our decision to manage *Acanthamoeba* keratitis medically. This case report indicated that real-time PCR is effective in detecting *Acanthamoeba* cases where cytopathologic examinations, including Fungiflora Y staining and culture, fail to indicate its presence. Clinical evaluations using real-time PCR in addition to routine cytopathologic examinations and culturing for *Acanthamoeba* detection would allow a more rapid and accurate diagnosis of *Acanthamoeba* keratitis to be made.

EPITHELIAL KERATITIS WITH SIZABLE PSEUDODENDRITE POSITIVE FOR VZV

Case

A 57-year-old woman with a history of anterior uveitis in the left eye that had resolved with treatment of topical and oral steroids reported having decreased vision.[2] Her visual acuity was 20/400, and a slit-lamp examination showed that she had keratitis with a large dendritic lesion (Fig. 2) and anterior chamber inflammation. She had no findings in the right eye. Epithelial scrapings for real-time PCR analysis were performed, and only VZV-specific DNA (4.9×10^4 copies per sample) was detected. This case was diagnosed as epithelial keratitis with sizable pseudodendrite of zoster sine herpete. The patient was treated with 2000 mg of valacyclovir hydrochloride daily for 2 weeks followed by 3% topical acyclovir (ACV) ointment 5 times daily for 8 weeks, 3 times daily for 8 weeks, and once daily for 8 weeks. Pseudodendrites and anterior uveitis improved, and VZV DNA at the recovery stage was undetectable by real-time PCR. The visual acuity returned to 20/20.

Typical VZV pseudodendrites have been reported to be small. However, we observed complicated atypical extensive VZV pseudodendritic keratitis in this case. This diagnosis was difficult to make because zoster sine herpete did not cause dermal findings or iris atrophy. Because toxic keratopathy should be included in the differential diagnosis of

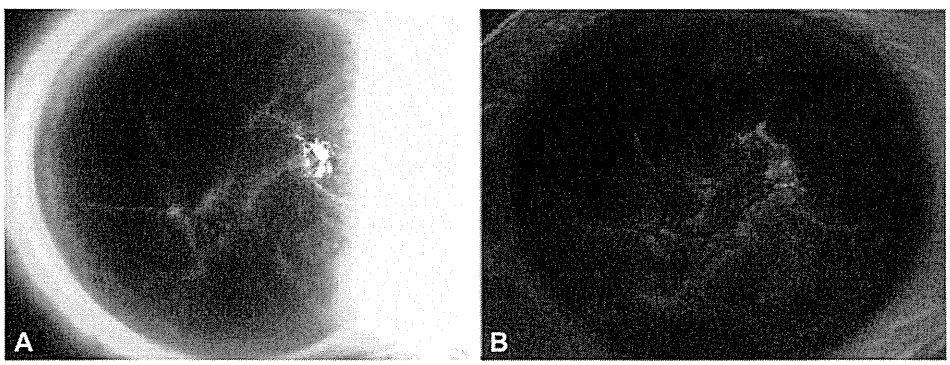

FIGURE 2. Slit-lamp photograph (A) and fluorescein stain (B). Large pseudodendrites are revealed. Reprinted from *Jpn J Ophthalmol.* 2009;53:548–549 with permission (Kandori et al[2]).

extensive pseudodendrites, it was difficult to treat them with sufficient antiviral agents without a definitive diagnosis of VZV because of the poor sensitivity to ACV. Thus, real-time PCR of VZV was important for the diagnosis of this case with atypical large pseudodendrites.

CORNEAL ENDOTHELIITIS POSITIVE FOR CMV

Case

A 58-year-old man, who underwent penetrating keratoplasty (PKP) in his left eye 5 months previously for keratouveitis and total bullous keratopathy, presented decreased vision in that eye [BCVA, 20/200; intraocular pressure (IOP), 14 mm Hg].[3] The slit-lamp examination indicated the presence of localized corneal edema with several keratic precipitates (KPs) (Fig. 3A). Because the edema was associated with minimal stromal infiltrates and endothelial dysfunction, corneal endotheliitis was diagnosed. The fundus, optic nerve, and contralateral eye were normal. The patient had no history of systemic disease. From these findings, corneal allograft rejection was suspected; however, steroid treatment was ineffective. Allograft rejection and herpetic corneal endotheliitis can have similar appearances. Resistance to rejection therapy suggested herpetic infection as another diagnostic option. Real-time PCR analysis for HHVs in the patient's aqueous humor was performed. Our real-time PCR procedure detected only CMV DNA (1.4×10^6 copies per milliliter). From these findings, this case was diagnosed as CMV corneal endotheliitis. The medications were switched to systemic and topical ganciclovir (GCV), an antiviral agent not specific for CMV, with a topical steroid (0.1% betamethasone). After the initiation of GCV treatment, corneal edema and KPs gradually disappeared (Figs. 3B, C). The CMV copy number gradually decreased in accordance with improved visual acuity and clinical appearance (Fig. 3D).

In a previous study to assess corneal scrapings and aqueous humor samples analyzed by PCR that were positive for CMV in patients with keratitis of an unknown origin, no cases of epithelial or stromal keratitis contained CMV DNA, and approximately one-quarter of corneal endotheliitis cases were positive for CMV. Thus, CMV should be included in the differential diagnosis of idiopathic corneal endotheliitis or graft edema after a PKP is performed, especially for bullous keratopathy. Because clinical remission after GCV may depend on the area of the normal endothelium, early diagnosis and therapy are important for CMV corneal endotheliitis.

CORNEAL ENDOTHELIITIS POSITIVE FOR HHV-7

Case

A 62-year-old man presented with a foreign-body sensation and decreased vision in his left eye.[4] At the first examination, BCVA was 20/400, and the IOP was 33 mm Hg in the affected eye. A slit-lamp examination showed severe corneal edema, ciliary injection, and KPs (Fig. 4A). He had no history of systemic diseases. Based on the ocular manifestation, idiopathic corneal endotheliitis with iridocyclitis was suspected; however, topical and systemic steroids (0.1% betamethasone 4 times per day and oral betamethasone 1 mg/d) and ocular antihypotensive therapy (0.5% timolol twice daily, oral acetazolamide 500 mg daily) were ineffective, resulting in the suspicion of a viral infection. Real-time PCR analysis for HHVs in the patient's aqueous humor could detect only HHV-7 DNA (4.1×10^5 copies per milliliter). These findings led to the diagnosis of HHV-7–related keratitis. The medications were replaced with topical 1% GCV, an antiviral agent for CMV and HHV-7, 6 times per day with a topical steroid (0.1% betamethasone 4 times per day). After GCV therapy was started, corneal edema and KPs were ultimately resolved

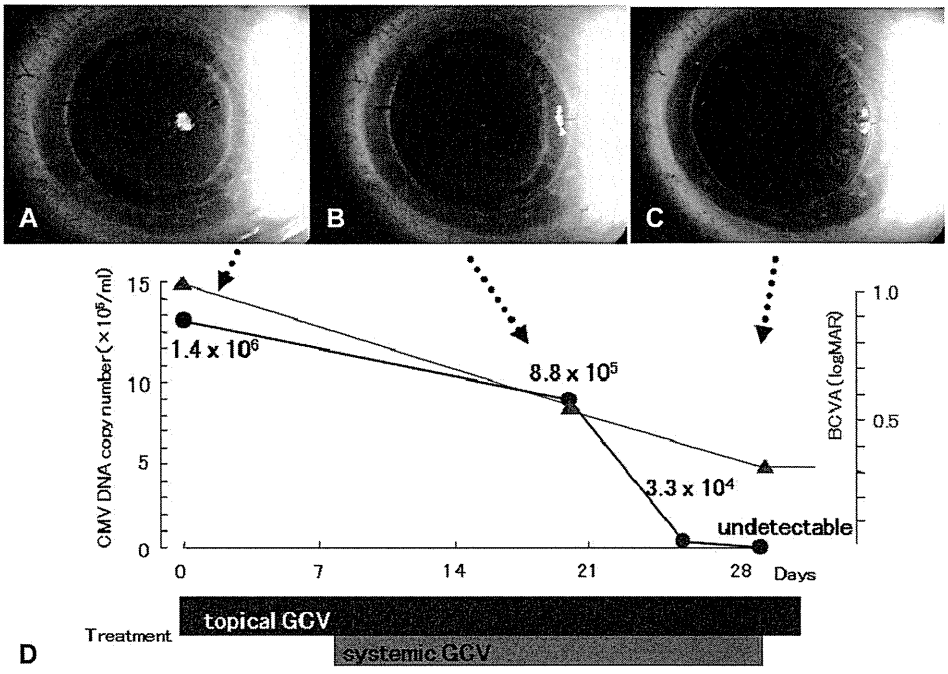

FIGURE 3. The relationship between therapeutic outcome with GCV and CMV copy number. A, Slit-lamp photograph before GCV treatment. Localized corneal edema (arrowheads) with several KPs was observed. B, Slit-lamp photograph 2 weeks after GCV treatment. Localized corneal edema gradually decreased (arrowheads) compared with (A). C, Slit-lamp photograph 4 weeks after GCV treatment. The corneal edema and KP are decreased. D, The CMV copy number gradually decreased in response to GCV and reached an undetectable level along with improved visual acuity and slit-lamp findings. Reprinted from Kandori et al[3] with permission from Elsevier.

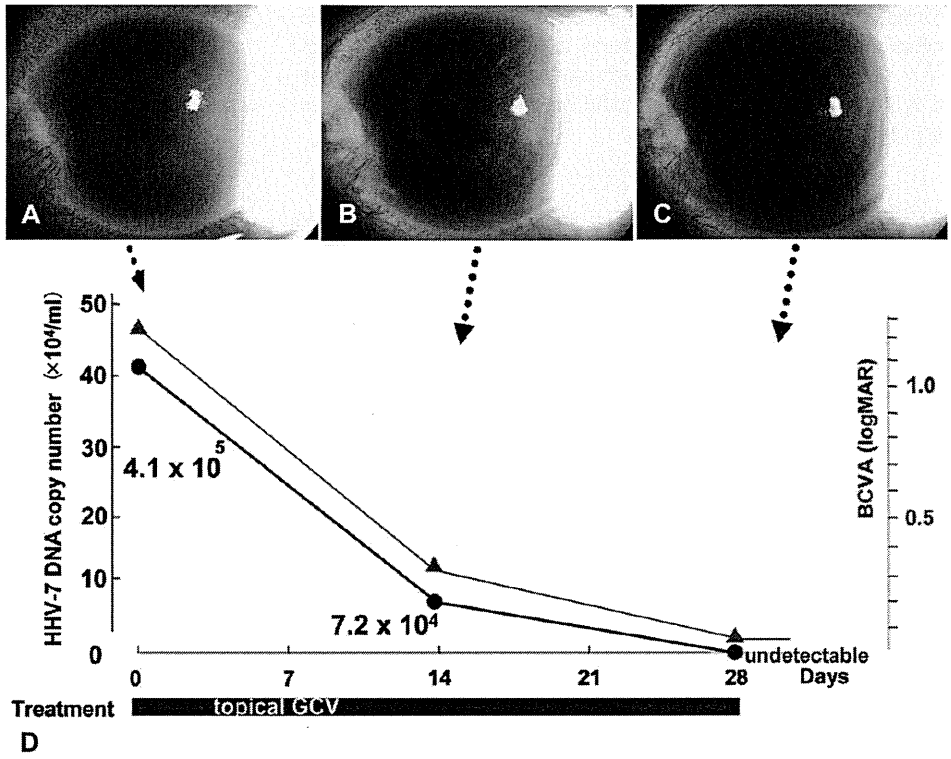

FIGURE 4. The relationship between therapeutic outcome with GCV and HHV-7 copy number. A, Slit-lamp photograph shows total corneal edema with KPs before GCV treatment (day 0). B, The corneal edema gradually decreased but localized edema remained 2 weeks after GCV treatment (day 14). C, The corneal edema and KPs were decreased 4 weeks after GCV treatment (day 28). D, The HHV-7 copy number gradually decreased in response to GCV and reached an undetectable level along with improved visual acuity and slit-lamp findings. Modified and reprinted from Inoue et al[4] with permission from the American Medical Association.

(Figs. 4B, C). The IOP gradually decreased to below 15 mm Hg without the use of any hypotensive agents. The BCVA recovered to 20/20 along with improved slit-lamp findings. The number of HHV-7 copies decreased after the start of the GCV therapy to an undetectable level and correlated with clinical improvement, and the antiviral therapy was terminated (Fig. 4D). The number of corneal endothelial cells in the affected eye decreased to 1052 cells per square millimeter during the recovery stage compared with 2432 cells per square millimeter in the unaffected eye.

To the best of our knowledge, this is the first report of real-time PCR–confirmed corneal endotheliitis positive for HHV-7 in the aqueous humor of an affected eye, and is also the first ocular manifestation related to HHV-7. Using PCR to detect HHV-7 did not necessarily mean that HHV-7 caused the clinical manifestations of corneal endotheliitis. However, in the current patient, topical antiviral therapy effective for HHV-7 improved the clinical status and decreased the HHV-7 load, indicating that HHV-7 may have been the causative agent of the corneal endotheliitis.

CORNEAL ENDOTHELIITIS POSITIVE FOR HHV-8

Case

A 56-year-old man, who underwent a PKP in his left eye 5 years previously for recurrent keratouveitis and total bullous keratopathy, presented with ocular pain and a sudden decreased vision in that eye (BCVA, 20/100; IOP, 25 mm Hg).[5] Examination showed severe localized corneal edema mainly in the graft, ciliary injection, KPs, and mild anterior chamber inflammation (Fig. 5A). He had diabetes mellitus and hypertension but not cancer or immunosuppressive disease. Based on ocular manifestations, corneal allograft rejection was suspected; however, topical and systemic steroids (0.1% betamethasone hourly and intravenous methylprednisolone hemisuccinate 125 mg 3 times) and ocular antihypotensive therapy (0.5% timolol twice daily, oral acetazolamide 500 mg daily) were ineffective. An aqueous humor sample subjected to our real-time PCR detected only HHV-8 DNA (2.5×10^5 copies per milliliter), suggesting that HHV-8 caused corneal endotheliitis after PKP; however, anti-HHV-8 treatment was unavailable. Despite allograft rejection therapy, corneal edema progressed to total bullous keratopathy and graft failure (Fig. 5B). Furthermore, HHV-8 copies did not decrease 4 weeks posttreatment (2.2×10^5 copies per milliliter). Because inflammation gradually resolved and the IOP decreased below 15 mm Hg untreated, antiallograft rejection therapy ended after 3 months when no HHV-8 copies were detected. The BCVA did not recover to 20/2000, and the corneal graft did not improve (Fig. 5C). The patient awaits corneal regrafting.

Although HHV-8 causes neoplastic disease, HHV-8–related clinical manifestations are not well defined. This case suggested that HHV-8 infection might play a role in corneal endotheliitis leading to graft failure. Investigations regarding specific anti-HHV-8 therapy or the latency of HHV-8 are required.

ACV-RESISTANT HERPETIC KERATITIS ESTIMATED BY CHANGES IN VIRAL DNA COPY NUMBER BEFORE AND AFTER TREATMENT

Case

A 37-year-old man had a history of herpetic keratitis in the left eye.[6] The herpetic keratitis resolved with periodic

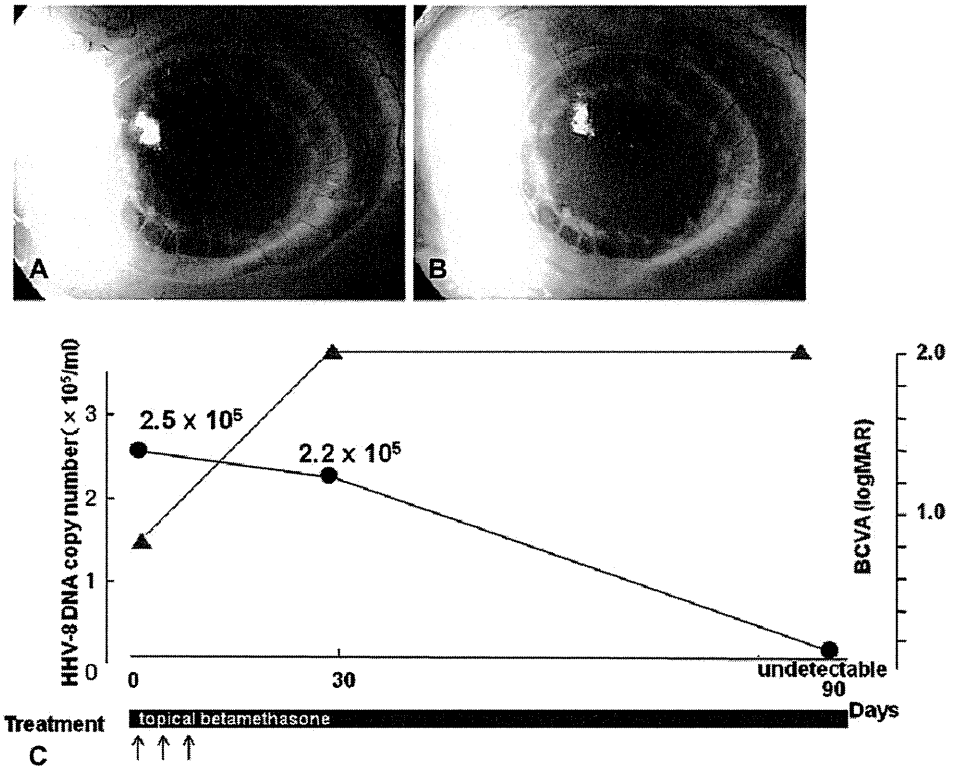

FIGURE 5. The relationship between the therapeutic outcome with allograft rejection therapy and HHV-8 copy number. A, Slit-lamp photograph shows localized corneal edema on a corneal graft with KPs (arrowheads) and severe infection before treatment (day 0). B, Slit-lamp photograph shows that corneal edema gradually progressed and resulted in total bullous keratopathy 4 weeks posttreatment (day 28). C, Although allograft rejection therapy continued, the corneal edema progressed gradually to total bullous keratopathy resulting in graft failure, and HHV-8 copies did not decrease 4 weeks posttreatment. When the HHV-8 copy number reached an undetectable level, the visual acuity and the clinical appearance of the bullous keratopathy 3 months after treatment (day 90) did not improve. The arrow indicates the administration of 125 mg of intravenous methylprednisolone hemisuccinate. Modified and reprinted from Inoue et al[5] with permission from the American Medical Association.

treatment of ACV ointment and a topical steroid. He had reported having a foreign-body sensation and redness in his left eye and periodically received topical antibiotics and a steroid for 1 month. His BCVA was 20/1000, and the IOP was 15 mm Hg in the affected eye. Slit-lamp examination showed epithelial erosion in the central cornea that manifested as geographic lesions with corneal infiltrates, moderate ciliary injection, and no anterior chamber inflammation (Fig. 6A). The

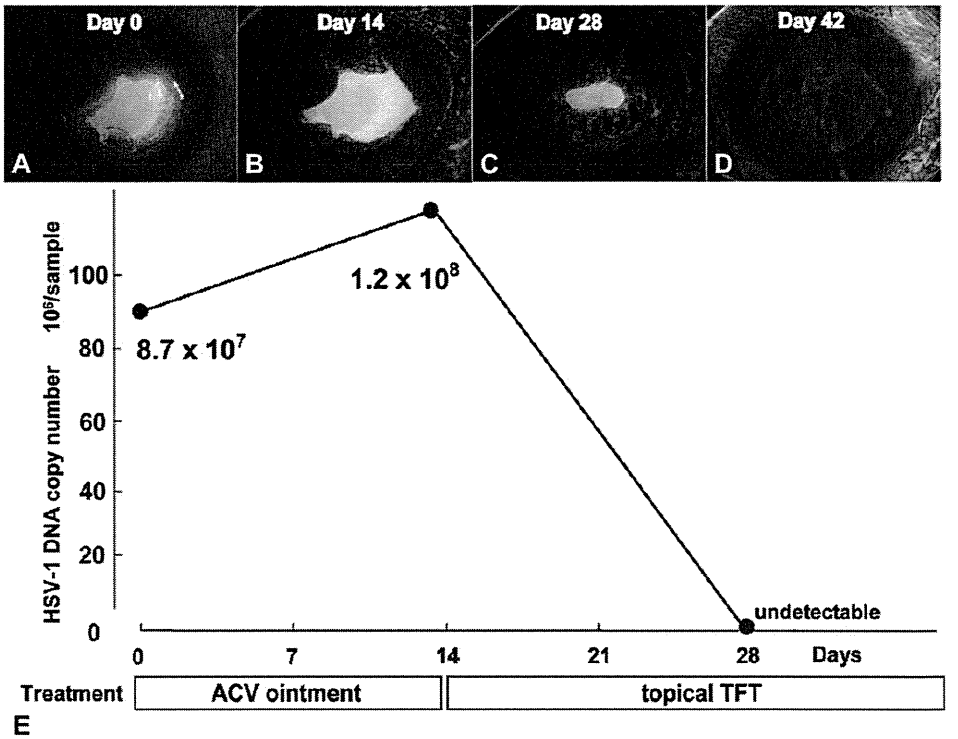

FIGURE 6. Relationship between the therapeutic outcomes with ACV and TFT antiviral therapy and HSV DNA copy numbers. A, Slit-lamp photograph with fluorescent staining shows geographic keratitis before topical ACV treatment (day 0). B, Slit-lamp photograph with fluorescent staining shows that geographic keratitis was not improved after topical ACV treatment (day 14). C, Slit-lamp photograph with fluorescent staining shows gradual lesion improvement after topical TFT therapy (day 28). D, Slit-lamp photograph with fluorescent staining shows the resolution of the lesions (day 42). E, Although the ACV treatment was continued, the geographic lesion was not improved, and HSV DNA copy numbers did not decrease. After topical TFT was substituted for ACV, the lesions slowly improved and healed, and the HSV DNA copy number became undetectable. Modified and reprinted from Inoue et al[6] with permission from the American Medical Association. ACV indicates acyclovir; TFT, trifluorothymidine.

sensitivity of the left cornea decreased to 10 mm compared with 60 mm in the right eye. A wide corneal scraping was performed for cytopathologic examination and culturing to detect pathogenic microorganisms such as bacteria, fungus or *Acanthamoeba*. Real-time PCR analysis was performed to detect HHVs. Light microscopy and culture findings were negative for any pathogens. The PCR results were only positive for HSV-1 DNA (8.7×10^7 copies per sample) (Fig. 6E). Based on the positive PCR result for HSV-1 DNA, the patient was suspected to have HSV keratitis and was treated with 3% topical ACV ointment 5 times per day for 2 weeks. However, the geographic lesions did not improve (Fig. 6B). A second epithelial scraping from the diseased lesions was obtained for real-time PCR analysis and cytopathologic examination and culturing, which resulted in the detection of HSV-1 DNA only (1.2×10^8 copies per sample) (Fig. 6E) without the presence of other pathogens. Because ACV is a specific anti-HSV agent, treatment can generally improve herpetic keratitis based on decreased HSV DNA copy numbers compared with that before treatment. However, the HSV viral load in this case did not decrease because of ACV treatment, and the only pathogen detected was HSV. Because these findings indicated that HSV detected in these lesions might be an ACV-resistant strain, we substituted topical 1% trifluorothymidine solution. The ocular pain and photophobia gradually decreased, and the lesions slowly improved (Fig. 6C). Real-time PCR of the epithelial scraping after the start of trifluorothymidine therapy did not detect HSV-1 DNA (Fig. 6E), and the keratitis was healed with corneal scarring (Fig. 6D).

Because the conventional determination of an ACV-resistant HSV strain is technically difficult and requires experience, complex skills, and time to obtain results, the application of real-time PCR to drug-resistant HSV keratitis is preferable because of its simplicity, high sensitivity, and speed. This estimation by real-time PCR is especially helpful when clinical signs and risk factors of herpetic infection are present along with detection of herpetic DNA, and the antiherpetic agent is ineffective.

CONCLUSIONS

A variety of pathogen DNA in keratitis can be assessed by real-time PCR, which is useful for both the diagnosis and monitoring of the clinical course. Real-time PCR has the advantages of high sensitivity and speed, and can identify other pathogens simultaneously from minute volumes of ocular samples for differential diagnosis. These advantages can assist ophthalmologists to make an early diagnosis and provide appropriate treatment for keratitis with complex clinical appearances.

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Genotypic Characterization of *Staphylococcus aureus* Isolates From Cases of Keratitis and Healthy Conjunctival Sacs

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Purpose: *Staphylococcus aureus* is the predominant pathogen that causes keratitis, and the rate of occurrence of drug-resistant *S. aureus* is increasing. However, little is known about its clinical epidemiology in Japan. This study was designed to characterize the genotypes of methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) isolates from keratitis and healthy conjunctival sacs.

Methods: Thirty keratitis isolates (19 MSSA and 11 MRSA) and 18 healthy conjunctival sac isolates (16 MSSA and 2 MRSA) obtained before cataract surgery were used. They were characterized by multilocus sequence typing. The prevalence of Panton–Valentine leukocidin was determined.

Results: In multilocus sequence typing analysis, ST5 and ST764 (ST5 variant) were the most frequently identified sequence types in MRSA strains from both keratitis and healthy conjunctival sacs. ST188 was the most frequently identified sequence type in the 19 MSSA isolates from keratitis (4 isolates, 21.1%), but was only identified in 1 of the 16 commensal MSSA strains. ST8 was found to be predominant among the 16 commensal MSSA strains (5 isolates, 31.3%). None of the isolates had Panton–Valentine leukocidin genes.

Conclusions: MRSA isolates from keratitis and healthy conjunctival sacs may have similar genotypic characteristics, but certain clones occur more often among MSSA isolates from keratitis than among commensal MSSA strains. These results suggest that specific MSSA lineages that possess genotypic characteristics can more effectively cause keratitis.

Key Words: *Staphylococcus*, MRSA, genotype, keratitis, microflora

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Infectious keratitis can progress rapidly with devastating consequences, including corneal scarring and loss of vision. Thus, it is imperative to identify this condition promptly and to begin an aggressive course of therapy to limit tissue damage. Because the severity of keratitis and response to treatment depend on the virulence or drug sensitivity of the causative agents, it is important to understand the various clinical and pathophysiological aspects of infectious keratitis caused by each pathogen. *Staphylococcus aureus* is the leading cause of infectious keratitis associated with contact lens wear, trauma, and corneal surgery.[1] It is also the leading cause of community- and hospital-acquired infections of the skin, soft tissue, bloodstream, and other sites. Moreover, *S. aureus* is a commensal organism of the adjacent skin and mucosa of the eye and nose, and can easily contaminate the surface of the eye. Thus, it is possible that commensal *S. aureus* could cause keratitis after the destruction of the ocular surface barrier, for example, because of contact lens use or trauma. However, little is known of the similarities between the characteristics of *S. aureus* isolates that cause keratitis and commensal isolates from the conjunctival sac.

When we consider the clinical aspects of keratitis caused by *S. aureus*, we should not ignore antibiotic resistance, which initially developed in hospitals but has since spread to the community, where the rates of occurrence of methicillin-resistant *S. aureus* (MRSA) are now approaching those of hospitals.[2,3] MRSA can cause keratitis as a serious complication of ocular surface disease or surgery.[4–8] Because antibiotics are used for ocular surface disease and ocular surgery, commensal MRSA could be selected in eyelids and conjunctival sacs and cause keratitis. Khan et al[9] reported that MRSA isolated from patients with keratitis possesses type II and III staphylococcal cassette chromosome mec (SCCmec) elements, which are detected in hospital-acquired MRSA (HA-MRSA). However, the genotype of MRSA isolated from ocular sites in the Japanese population has not been well documented. Thus, it is important to investigate and compare the epidemiology of MRSA and methicillin-sensitive *S. aureus* (MSSA) isolated from patients with keratitis and from healthy conjunctival sacs.

Multilocus sequence typing (MLST) has been used to investigate the phylogenetic relationships of a variety of bacterial pathogens, including *S. aureus*.[10] It can be used to elucidate evolutionary relationships among strains and to identify ancestral genotypes, and to predict the patterns of divergence within groups of related genotypes. E-burst, an MLST

program, can be used with multilocus data to define groups or clonal complexes (CCs) of related isolates derived from a common ancestor, the patterns of descent linking them, and the ancestral genotype. In this study, *S. aureus* isolates from keratitis and healthy conjunctival sacs, including MSSA and MRSA, were compared using MLST.

MATERIALS AND METHODS

Bacterial Isolates

Thirty *S. aureus* keratitis isolates and 18 healthy conjunctival sac isolates were used. In keratitis isolates, 16 isolates were obtained from the Ehime University Hospital, and another 14 isolates were collected from various places in Japan and stocked as reported in a Japanese multicenter study.[11] The healthy conjunctival sac isolates were isolated from patients 3 days before their cataract surgery in the Ehime University Hospital, Okamoto Eye Clinic, and Machida Hospital, which were on the same island, Shikoku, and geographically close. Patients with cataract had not used mydriatic agents, antibiotic eye drops, and systemic antibiotics before collection of the samples, and their conjunctivas and facial skin were not inflamed. The isolates included methicillin-sensitive and methicillin-resistant strains.

Multilocus Sequence Typing

S. aureus was cultured in brain heart infusion broth at 37°C, and DNA was extracted using a DNA Mini Kit (Qiagen, Valencia, CA). Polymerase chain reaction (PCR) fragments of 7 housekeeping genes were amplified using the following primers: carbamate kinase (*arcC*), *arcC*-F (5′-TGT GAT GAG CAC GCT ACC GTT AG-3′) and *arcC*-R (5′-TCC AAG TAA ACC CAT CGG TC TG-3′); shikimate 5-dehydrogenase (*aroE*), *aroE*-F (5′-CAT TGG ATT ACC TCT TTG TTC AGC-3′) and *aroE*-R (5′-CAA GCG AAA TCT GTT GGG G-3′); glycerol kinase (*glpK*), *glpK*-F (5′-CATCACCACGGTCAAAACATGC-3′) and *glpK*-R (5′-CAG GTC GTC CAA TCT ATC ACG C-3′); guanylate kinase (*gmk*), *gmk*-F (5′-TCG ATT CTT AGC GAG TTC AAC C-3′) and *gmk*-R (5′-CCT TCA GGT GTT GGA AAG GG-3′); phosphate acetyltransferase (*pta*), *pta*-F (5′-TAC TGC ATC GTA TCC ACC TAA ACG-3′) and *pta*-R (5′-TGG TGC TGC ACA TTC TAC TGG AG-3′); triosephosphate isomerase (*tpiA*), *tpiA*-F (5′-CCA CCA TAT TGA ATA CGT GTA GCG-3′) and *tpiA*-R (5′-GCT TAC TTT GAA GAA AGC GGT G-3′); and acetyl coenzyme A acetyltransferase (*yqi*), *yqi*-F (5′-TGC TGG ACG GAG TTG TGC TAA C-3′) and *yqi*-R (5′-ATC CTG CTC GTA TTG CTG CG-3′) (http://www.mlst.net). Nucleotide sequences were determined for both the strands by direct, automated sequencing of the PCR products using an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were compared with those of known alleles for each locus via the MLST website (http://www.mlst.net). For each isolate, a 7-digit allelic profile that defined a sequence type (ST) was established. The clustering of STs was analyzed using the eBURST (electronic Based Upon Related Sequence Types) algorithm[12] (www.eburst.mlst.net).

PCR Identification of SCCmec Types and Panton–Valentine Leukocidin

SCCmec type I–IV elements in MRSA strains were identified by multiplex PCR amplification of SCCmec regions.[13] Genes for Panton–Valentine leukocidin (PVL) were identified by simplex PCR amplification using primers described elsewhere.[14]

RESULTS

MLST Reveals Genetic Associations Between Commensal Conjunctival Sac Isolates and Keratitis Isolates

The MLST of all 48 *S. aureus* strains analyzed in this study identified 17 different STs (Table 1). Phylogenetic analyses of the STs of all the strains in this study revealed close relationships among both commensal and keratitis isolates of *S. aureus* (Fig. 1). Among the 18 commensal isolates, 10 different STs were identified, 1 of which (ST2561) was new. ST8 had the highest prevalence among commensal isolates (30%). Fourteen different STs were found in keratitis isolates, with ST5, ST764, and ST188 being the most common (Table 1). MRSA strains comprising 11 keratitis isolates and 2 commensal isolates had 4 different STs, 1 of which (ST2560) was new. ST5 and ST764, which are variants of the globally disseminated ST5 lineage, were predominant among MRSA strains, with a prevalence of 46.2% and 38.5%, respectively. ST5 or ST8 was identified in 2 commensal MRSA strains (Table 1). The 19 MSSA keratitis isolates had 11 different STs, with ST188 having the highest prevalence (21.1%), whereas the 16 MSSA commensal isolates had 10 different STs, with ST8 having the highest prevalence (31.3%). The population structure was determined by the eBURST analysis of MRSA STs present in the *S. aureus* MLST database as of April 11, 2013. The STs of the isolates tested in our study are consistent with the STs with high frequencies in the database. Aggregation of STs by eBURST identified a distinct CC (Fig. 2). CC5 includes ST5 and ST764 and was present in 11 MRSA isolates and 1 MSSA isolate, including 10 keratitis isolates and 2 commensal isolates. CC8 includes ST8 and ST623 and was present in 3 keratitis isolates and 6 commensal isolates. ST188, ST81, and ST25 were closely related in the eBURST analysis and showed a high prevalence among keratitis isolates (6/7, 85.7%).

SCCmec Types and Panton–Valentine Leukocidin in MRSA Isolates

All ST5 and ST764 isolates had SCCmec type II elements. By contrast, no SCCmec elements were detected in ST8 and ST2560 by multiplex PCR. None of the isolates in this study possessed PVL genes.

DISCUSSION

S. aureus is frequently isolated from healthy conjunctival sacs, with a prevalence rate of approximately 10%.[15,16] Thus, commensal *S. aureus* strains that colonize the conjunctival sac and skin adjacent to the eye could contaminate the

TABLE 1. Distribution of Commensal and Keratitis Isolates by MLST

		Sequence Type																	
Source	**Organisms**	**188**	**8**	**5**	**764**	**121**	**15**	**45**	**12**	**25**	**59**	**20**	**81**	**623**	**96**	**508**	**2560**	**2561**	**Total**
Keratitis	MSSA	4	2	—	—	2	2	2	2	1	1	1	1	1	—	—	—	—	19
	MRSA	—	—	5	5	—	—	—	—	—	—	—	—	—	—	—	1	—	11
Healthy conjunctival sac	MSSA	1	5	1	—	1	—	2	2	—	—	1	—	—	1	1	—	1	16
	MRSA	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2

ocular surface, leading to keratitis. To understand the pathogenesis of *S. aureus* keratitis, the relationship between commensal and keratitis-causing strains must be investigated. MLST is a useful technique for assessing the genetic diversity between groups.

In this study, MLST analysis revealed the genetic diversity among MSSA strains, but showed that ST8 is predominant in commensal MSSA isolates, and ST188 is predominant in MSSA keratitis–causing strains. Moreover, eBURST analysis showed that certain clones (T188, ST81, and ST25) with similar genetic characteristics have a high prevalence in MSSA keratitis isolates. Some molecular characterization studies of MSSA have demonstrated a marked genetic diversity among MSSA strains.[17,18] However, Miller et al[19] reported that ST8 is predominant among MSSA strains in the United States. Because the STs of keratitis and conjunctival sac isolates had high frequencies in all the strains registered in the database, certain clones exist in ocular sites and in other sites—such as the skin. A recent study from Malaysia showed that MSSA strains from community and clinical sources are genetically diverse, but that ST188 occurs more often among clinical isolates than among carriage isolates, similar to our findings.[20] These observations suggest that certain clones may be more virulent in the cornea than others. Virulence factors in *S. aureus* keratitis have been investigated in vitro and in vivo. Wall teichoic acids and fibronectin-binding protein play critical roles in the adhesion and internalization into corneal epithelial cells of *S. aureus*.[21,22] Bacterial toxins,

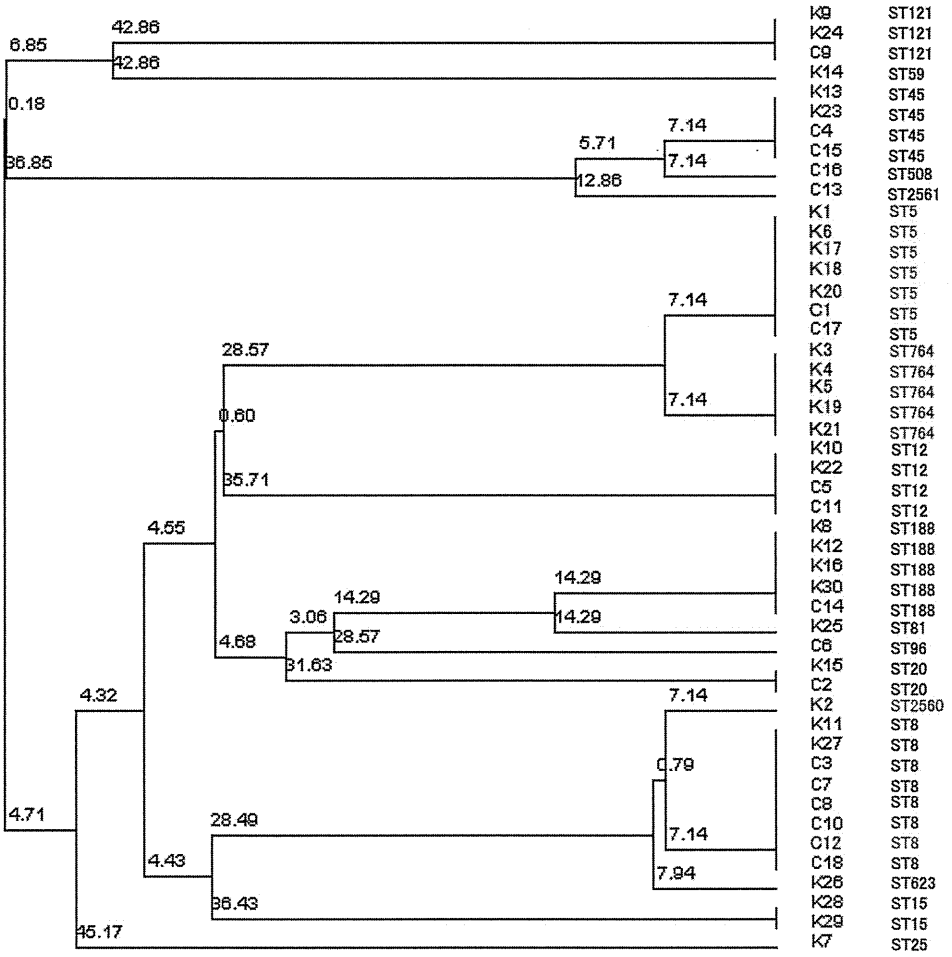

FIGURE 1. Phylogenetic relationships between *S. aureus* keratitis and commensal isolates, as determined by performing the MLST analysis. Unweighted pair group method with arithmetic mean (UPGMA) tree showing the genetic relationships between the merged sequences of 7 housekeeping gene fragments from *S. aureus* isolates. Thirty-five MSSA strains (black) and 13 MRSA strains (red) are represented. For each isolate, the source (K, keratitis; C, conjunctival sac) and number are shown. The scale indicates the levels of genetic relatedness within this set of isolates.

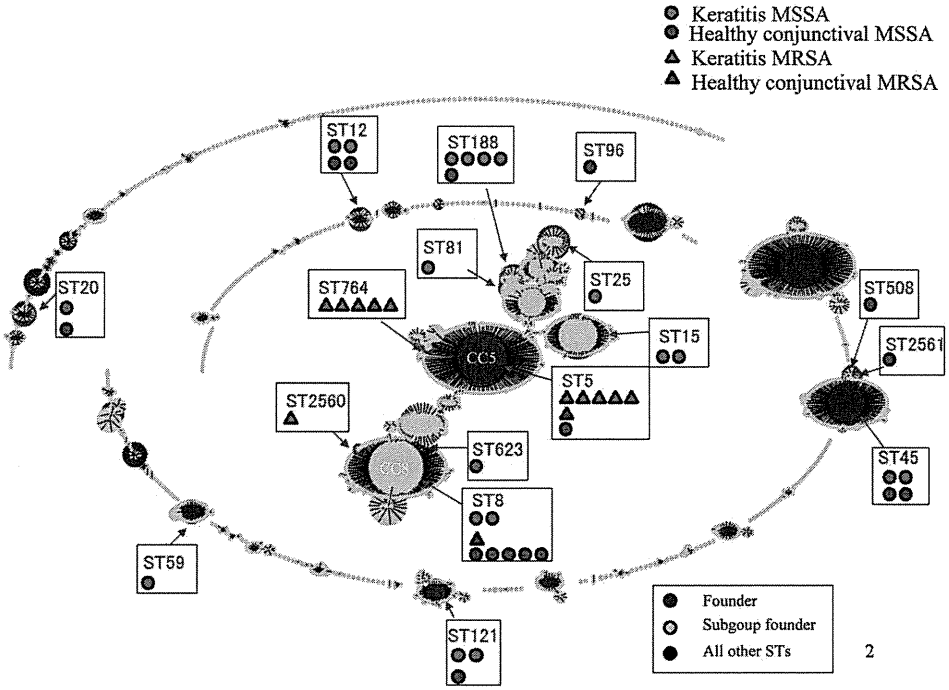

FIGURE 2. Population structure of the *S. aureus* MLST database as of April 2013, depicted graphically using eBURST V.3. Each circle represents an individual ST. Circle sizes indicate the frequency of a particular ST within the database. The eBURST algorithm draws a line between all STs that share 6/7 alleles (single locus variants, SLVs), and groups together sequence types that share at least 5/7 alleles (double locus variants, DLVs) into clusters referred to as CCs. Blue circles within CCs represent "founders," defined as the ST with the greatest number of SLVs, and typically represent the most prevalent ST within a CC. Yellow circles denote "subgroup founders" within a given CC. The presence of isolate STs is shown as follows: green circle, keratitis MSSA; red circle, healthy conjunctival MSSA; green triangle, keratitis MRSA; and red triangle, healthy conjunctival MRSA.

especially alpha-toxin, can mediate corneal disease in mice.[23] These factors could be activated in some clones. Further investigation is needed to determine the factors in ST188 that mediate its virulence in keratitis.

Although fluoroquinolones and cephalosporins are widely used for the treatment and prevention of *S. aureus* keratitis, they are less efficacious against MRSA.[24] Thus, keratitis caused by MRSA occurs after ocular surface surgery in which fluoroquinolones and cephalosporins are used for the prophylaxis of infection.[4–8] MRSA was traditionally associated with healthcare facilities, but its prevalence has reportedly increased in otherwise healthy patients without identified risk factors. Methicillin resistance in *S. aureus* is on the rise in the community in a large part because of the proliferation of the infectious USA300 lineage.[3] A population review conducted in 3 communities showed that the annual incidence of community-acquired MRSA (CA-MRSA) during 2001 to 2002 was 18 to 25.7/100,000[2]; most CA-MRSA isolates were associated with clinically relevant infections, and 23% of the patients required hospitalization. Generally, the virulence of CA-MRSA against human tissues is higher than that of HA-MRSA because CA-MRSA has virulence determinants such as alpha-toxin and phenol-soluble modulins, and the acquisition of PVL, which can destroy white blood cells and cause extensive tissue necrosis.[3] Thus, it is possible that healthy patients without risk factors have keratitis caused by MRSA, so it is important to determine whether CA-MRSA is present in those with keratitis. Hsiao et al[25] reported the isolation of both CA-MRSA and HA-MRSA from sites with ocular infections, including keratitis. However, they characterized HA-MRSA and CA-MRSA using patient information. To better define HA-MRSA and CA-MRSA, genetic analysis of isolates should be performed. Enright et al investigated MRSA isolates obtained between 1961 and 1999 from 20 countries using both SCCmec typing and MLST. Five CCs were found among the population, and strains with the same ST harbored different SCCmec types.[26] Among these clones, the New York/Japan clones, which have ST5 and SCCmec type II elements, are among the most globally disseminated HA-MRSA lineages.[27,28] In our study, ST5/SCCmec type II clones were found to be predominant in commensal and keratitis MRSA isolates. ST764/SCCmec type II elements were isolated, as were ST5/SCCmec type II clones. ST764, an ST5 variant, was isolated from children in the Japanese community.[29] Interestingly, ST764 carries CA-MRSA virulence determinants because it has mobile genetic elements that increase virulence, despite having no PVL.[30] This study shows that keratitis and conjunctival sac isolates have characteristics of both HA-MRSA and CA-MRSA. The findings confirm the potential emergence of MRSA keratitis in community settings in Japan. Further investigation of the differences between ST5 and ST764 in terms of virulence against corneal tissue is needed. MLST analysis of *S. aureus* isolated from keratitis was also performed in India,[31] and ST772/SCCmec type V, a CA-MRSA clone, predominated in severe keratitis cases. ST772/SCCmec type V has PVL, and could be related to the severity of keratitis. Although none of the isolates in our study possessed PVL genes, epidemiological surveillance of CA-MRSA with PVL should be conducted on a regular basis.

In conclusion, it is observed that certain clones occur more often among MSSA keratitis isolates than among commensal isolates. Moreover, CA-MRSA and HA-MRSA clones were detected in both keratitis and conjunctival sac isolates.

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ORIGINAL ARTICLE

Prospective Clinical Evaluation of 1.5% Levofloxacin Ophthalmic Solution in Ophthalmic Perioperative Disinfection

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Abstract

Purpose: Gram-positive cocci and *Propionibacterium acnes* are widely reported agents of infectious postoperative endophthalmitis. This multicenter study was conducted to evaluate the eradication effectiveness and safety profile of levofloxacin 1.5% ophthalmic solution (LVFX 1.5%) in use for perioperative disinfection.
Methods: Patients who were scheduled for cataract surgery were enrolled. The perioperative regimen of LVFX 1.5% was administered 3 times daily as follows: preoperative 3 days; the day of surgery (in the morning, 1 h before surgery, and immediately after surgery); and postoperative 2 weeks. Conjunctival sac scrapings were collected 3 times in the observation period; before preoperative administration, before iodine eyewash on the day of surgery, and after completion of postoperative administration. Isolated and identified microbial strains were assessed for antibacterial susceptibly.
Results: One hundred patients were enrolled and data were obtained from 96 patients (mean age, 72.7±8.9 years). The preoperative eradication rate was 86.7% in total microbes. In case of gram-positive cocci, the preoperative eradication rate was 100%, even though there were LVFX-registrant methicillin-resistant *Staphylococcus aureus* and methicillin-resistant coagulase-negative *Staphylococcus*, which had a high minimum inhibitory concentration against LVFX, such as 32 μg/mL. On the other hand, that of *P. acnes* was 78.3%. No acquired drug resistance was suspected in all strains. Adverse drug reactions occurred in 4.2% patients, and all were slight.
Conclusions: For ophthalmic perioperative disinfection, the LVFX 1.5% ophthalmic solution showed a good safety profile, and critical eradication of gram-positive cocci, including the fluoroquinolone-resistant strains.

Introduction

Infectious postoperative endophthalmitis is a rare, but serious intraocular complication of cataract surgery that may lead to loss of visual acuity or blindness.[1,2] The incidence are reported to be between 0.025% and 0.052%.[3–6] Bacterial flora found in the external eye, specifically gram-positive cocci, such as *Staphylococcus aureus*, coagulase-negative *Staphylococcus* (CNS), *Enterococcus faecalis*, and *Propionibacterium acnes* have been widely studied and reported as key agents of postoperative endopthalmitis.[7–11] In addition, many of these strains were detected in conjunctival sac scrapings from patients who were scheduled to have cataract surgery.[8] Thus, disinfection of these gram-positive cocci and *P. acnes* in the conjunctival sac is important in preventing postoperative endophthalmitis.

According to the assessment of the clinical evidence strength of endophthalmitis prophylactic interventions by Ciulla et al.,[12] ophthalmic perioperative disinfection by the administration of an antibiotic ophthalmic solution was concluded to be probably relevant, but could not be definitely related to clinical outcome.[12] However, several reports investigated culture in the conjunctival sac before administration of an antibiotic ophthalmic solution and estimated eliminating conjunctival microorganisms before surgery.[7,13,14] Thus, reduction of microflora preoperatively could be effective to prevent endophthalmitis, and the eradication rate of the antibiotic ophthalmic solution could be one of the indices for prophylaxis of endophthalmitis.

The fluoroquinolone antibacterial ophthalmic solution has been widely used for perioperative disinfection because of its broad spectrum of antibacterial activity.[8] Inoue et al. assessed the positive microbes at each prophylactic intervention (pretreatment, after preoperative administration of antibiotics, after eyewash with diluted iodine compounds, and after surgery) through the cataract perioperative duration.[8]

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[3]Okamoto Eye Clinic, Matsuyama, Japan.

It was concluded that a 3-day preoperative administration of the levofloxacin 0.5% ophthalmic solution and an eyewash with diluted iodine compounds were effective for preoperative disinfection, however, *Staphylococcus epidermidis* and *P. acnes* were detected after the 3-day preoperative administration.[8] Also, other fluoroquinolone antibacterial ophthalmic solutions could not eradicate the CNS and *P. acnes* completely.[15,16] In addition, there were several concerning reports suggesting an increase in fluoroquinolone-resistant bacterial flora found in the external eye,[17] and the drug-resistance development of CNS caused by the topical fluoroquinolones.[18–20] Thus, the possibility of a lower antibiotic effect of topical fluoroquinolone is concerned in ophthalmic perioperative eradication. An antibiotic ophthalmic solution, which penetrates well into the ocular tissue and exceeds a higher concentration than the minimum inhibitory solution of antibiotics against a microbe, may offer effective eradication of microflora and reduction of susceptibility toward endophthalmitis.

The levofloxacin 1.5% ophthalmic solution (Cravit® ophthalmic solution 1.5%; Santen Pharmaceutical Co., Ltd., Osaka, Japan) was marketed in Japan in 2011. In contrast to other fluoroquinolone solutions, the levofloxacin solution has been shown to penetrate to the bulbar conjunctiva,[21] and hence is expected to be more effective for the eradication of microflora in the conjunctival sac. This multicenter prospective study was planned to evaluate the efficacy and safety of LVFX 1.5% in ophthalmic perioperative disinfection.

Methods

Design

This prospective open-label, multicenter study was performed in 3 clinical facilities and conducted in accordance with the Declaration of Helsinki (Amended in October 2008) and Ethical Guidelines for Clinical Studies (Amended in July 2008, Ministry of Health, Labour and Welfare, Japan). This study was registered in JAPIC-CTI (no. JapicCTI-111691, www.clinicaltrials.jp/user/ctiMain_e.jsp).

Participants and eligibility

Patients who were ≥20 years old and scheduled for cataract surgery were eligible for participation. Sex of patients and whether the patient was treated as an outpatient or an inpatient did not influence the patient selection. In cases where both eyes were scheduled for surgery, the eye that was scheduled for cataract surgery on an earlier date was selected for this study to avoid any sympathetic response.

Exclusion criteria were as follows: hypersensitivity to fluoroquinolones or a history of serious adverse reaction to fluoroquinolones; suspected bacterial, fungal, or viral infection; use of antibiotics (including eye drops) or immunosuppressive drugs (including steroids) within 1 month before the initiation of preoperative administration of LVFX 1.5%; surgery scheduled for both eyes on the same day; hospitalization for reasons other than cataract surgery; poorly controlled underlying disease or complications; being pregnant or nursing, having childbearing potential; use contact lenses during the observational period; or history of eye surgery for microbiological evaluation.

Intervention

Patients were enrolled in the study after they provided written informed consent. The day of cataract surgery was defined as day 0. Patients were administered the LVFX 1.5% ophthalmic solution as follows: before the cataract surgery (day −3 to day −1) 3 times a day; the day of cataract surgery (day 0) once in the morning, 1 h before surgery, and immediately after surgery; and 3 times a day for 2 weeks after the surgery (day 1 to day 13). This preoperative regimen was based on the protocol for a prospective randomized multicenter clinical study of preoperative disinfection of the conjunctival sac using levofloxacin and iodine compounds, which was conducted by the Japanese Association for Ocular Infection.[8]

From the initiation of preoperative LVFX 1.5% administration on day −3 until the conjunctival sac scraping before the iodine eyewash on day 0, concomitant use of any antibiotics (including eye drops) or immunosuppressive drugs (including steroids) was prohibited. From the termination of cataract surgery on days 0 to 14, there was no limitation on the use of drugs necessary for the cataract surgery. Concomitant treatment that would interfere with the evaluation of effectiveness and safety was prohibited through the observational period, including the use of contact lenses.

Conjunctival sac scrapings for bacteriological examination were collected 3 times in the observation period as follows: before LVFX 1.5% administration on day −3, before the iodine eye wash on day 0, and after the completion of postoperative administration on day 14. These scrapings were collected by wiping the inferior palpebral conjunctiva back and forth once with a swab moistened with sterile physiological saline, after anesthetizing the ocular surface with preservative-free ophthalmic solution. Subsequently, the swab was preserved onto media in a sealed tube (SEEDTUBE Eiken™; Eiken Chemical Co., Ltd., Tokyo, Japan) at 4°C before shipping to the microbial research site.

Ophthalmological examination was performed using slit-lamp microscopy and fluorescein staining for the anterior corneal epithelium 3 times with the following timing: before the conjunctival sac scraping on day −3, before the surgery on day 0, and before the conjunctival sac scraping on day 14.

Identification and antibacterial susceptibility of microbes

Microbial strains were isolated and identified from the conjunctival sac scraping samples. CHROMagar™ orientation plates (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan), 5% sheep blood agar M58 plates (Eiken Chemical Co., Ltd.), and Chocolate II agar plates (Nippon Becton Dickinson Company, Ltd.) were incubated for 40–48 h at 35°C to isolate aerobic microbes. Plates of Anaero Columbia agar with rabbit blood (Nippon Becton Dickinson Company, Ltd.) were incubated for 60–72 h at 35°C to isolate anaerobic microbes.

Antibacterial susceptibility was assessed as follows: the minimum inhibitory concentration (MIC) was determined using the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI).[22–25] *S. aureus* was considered to be methicillin resistant if its MIC against oxacillin (MPIPC) was ≥4 μg/mL. CNS was considered to be methicillin resistant, if the MIC against MPIPC was ≥0.5 μg/mL. MIC_{50} and MIC_{90} were calculated in case the number of the strain was 10 or more.

Genetic testing for the region of 16s ribosome was performed when a strain was suspected to acquire resistance to LVFX. In this study, genetic testing was performed for the identification of 2 strains of *Corynebacterum* species (sp) cultured from 1 patient as described.[26]

Analyses

The following rates were calculated to evaluate the effectiveness. The primary endpoint of this study was preoperative eradication rates.

(1) Positive culture rate: the number of patients having a positive culture divided by the number of patients who had conjunctival sac scraping at each scraping point (days −3, 0, and 14).
(2) Preoperative eradication rate (primary endpoint): number of patients having a positive culture on day −3 and a negative culture on day 0 divided by the number of patients having a positive culture on day −3.
(3) Preoperative positive conversion rate: number of patients having a positive culture on day 0 and having a negative culture on day −3 divided by the number of patients showing a negative culture on day −3.
(4) Preoperative microbial substitution rate: number of patients with positive culture on day 0, but in whom the detected microbes were different from the microbes detected on day −3, divided by the number of patients having a positive culture on day −3.

JMP version 9.0.2 (SAS Institute, Inc., Cary, NC) was used for statistical analyses. The Student's *t*-test, Wilcoxon rank sum test, and McNemar's test were performed for statistical comparison, and *P*-values < 0.05 were considered statistically significant.

For assessment of the safety of LVFX 1.5%, case number and incidence rates of adverse drug reactions were evaluated.

Results

Study populations

From December 2011 to April 2012, 100 patients were enrolled in this study. The data available for analyses were obtained from 96 eyes of 96 patients. Four patients were excluded from the assessment because of the use of prohibited concomitant drugs before the cataract surgery by 3 patients, and an error of conjunctival sac scrapings on day 0 in 1 patient. Subsequently, 2 patients were excluded from assessment on day 14 because these patients used a prohibited drug from day 8.

Characteristics of patients

The mean age of 96 patients was 72.7±8.9 years, and the age range was 38–89 years. 41.7% (40/96 patients) were men and 58.3% (56/96 patients) were women. About 90.6% (87/96 patients) were outpatients and 9.4% (9/96 patients) were inpatients.

Positive culture rates and preoperative eradication rates in total microbes

The positive culture rates were 78.1% (75/96 patients) on day −3, 11.5% (11/96 patients) on day 0, and 11.7% (11/94 patients) on day 14 (Fig. 1). The rates on days 0 and 14 were significantly reduced from the rate on day −3 (both were $P < 0.0001$ using the McNemar's test).

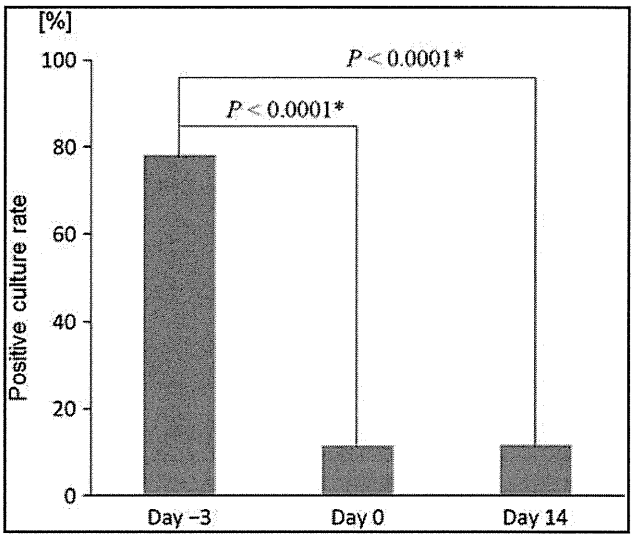

FIG. 1. Positive culture rates on days −3, 0, and 14. The positive culture rates on days 0 and 14 reduced significantly from day −3 ($P < 0.0001$). *McNemar's test.

The preoperative eradication rate was 86.7% in total microbes (65/75 patients, Fig. 2). The preoperative positive conversion rate was 4.8% (1/21 patients); *P. acnes* was cultured from this patient. The preoperative microbial substitution rate was 6.7% (5/75 patients). From these 5 patients, *P. acnes* was cultured from 3 patients, *E. faecalis* was cultured from 1 patient, and *Corynebacterium striatum* was cultured from 1 patient. Endophthalmitis was not observed in any patient throughout the observational period.

Preoperative eradication rates of each microbe

Table 1 summarizes the microbes cultured from 96 patients on day −3. The isolated aerobic gram-positive cocci

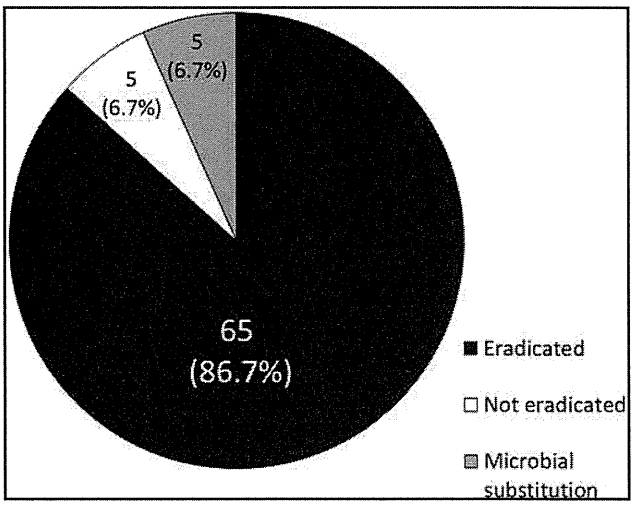

FIG. 2. Results of the 3-day preoperative eradication in 75 patients, who showed positive microbes on day −3. Preoperative eradication rate was 86.7%.

TABLE 1. PREOPERATIVE MICROBIOLOGICAL ERADICATION RATES OF EACH SPECIES BY LVFX 1.5%

Species	*Isolated strains*	*Eradicated strains*	*Preoperative eradication rates (%)*
Gram-positive coccus	55	55	100
Staphylococcus aureus	8	8	100
MSSA	(6)	(6)	100
MRSA	(2)	(2)	–
CNS	41	41	100
MSCNS	(16)	(16)	100
MRCNS	(25)	(25)	100
Streptococcus sp	3	3	–
Enterococcus faecalis	3	3	–
Corynebacterium sp	42	40	95.2
Gram-negative rods	7	7	100
Propionibacterium acnes	23	18	78.3

–, Preoperative eradication rate was not calculated if there were less than 5 strains.

MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; MRCNS, methicillin-resistant coagulase-negative *Staphylococcus*; MSCNS, methicillin-sensitive coagulase-negative *Staphylococcus*; LVFX 15%, levofloxacin 1.5% ophthalmic solution.

included coagulase-negative *Staphylococci* (41 strains, including *S. epidermidis, Staphylococcus hemolytics*, and *Staphylococcus lentus*), *S. aureus* (8 strains), *E. faecalis* (3 strains), and *Streptococcus* sp (3 strains, including Group G *Streptococcus* and α-hemolytic *Streptococcus*). Their preoperative eradication rate was 100% (55/55 strains). The preoperative eradication rates of *Corynebacterium* sp was 95.2% (40/42 strains). The preoperative eradication rate of aerobic gram-negative rods was 100% (7/7 strains), which included *Enterobacter cloacae, Serratia marcescens, Pantoea* sp, *Pseudomonas fluorescens, Sphingomonas paucimobilis*, and *Prophyromonas asaccharolytica*. The preoperative eradication rate of *P. acnes* was 78.3% (18/23 strains).

Result of bacteriological examination and MIC of positive strains

The results of bacteriological examinations on each day of collecting the conjunctival sac scraping (days −3, 0, and 14) are summarized in Table 2. For example, the result "+ − −" means that "Conjunctival sac microbes were positive on day −3 and negative on days 0 and 14." The MIC against LVFX of gram-positive cocci on day-3 are summarized in Table 3.

Evaluation of gram-positive cocci demonstrated that there were no microbes on day 0, which were positive on day −3. Even though there were LVFX-resistant methicillin-resistant *S. aureus* (MRSA) and LVFX-resistant methicillin-resistant coagulase-negative *Staphylococcus* (MRCNS), which maximum MIC were 32 μg/mL, they were all negative on day 0. On day 14, 1 strain of *S. epidermidis* and 1 strain of *Staphylococcus capitis* were positive. These 2 strains were LVFX-sensitive strains, because the MIC against LVFX was 0.25 μg/mL. However, on day 0, 1 strain of *E. faecalis* was positive by microbial substitution, which MIC was 64 μg/mL.

Evaluation of *Corynebacterium* sp, demonstrated that there was a LVFX-resistant strain, which was positive on days −3, 0, and 14 in 1 patient with a complication of nasolacrimal duct obstruction, which MIC against LVFX were all 128 μg/mL. In addition, there was a microbial substitution in 1 patient with a positive *Corynebacterium macginlery* on day −3, and as *C. striatum* on day 0. One strain of positive *Corynebacterium* sp on day 14 was LVFX sensitive, which was negative on days −3 and 0.

There were no positive gram-negative rods on day 0, but 4 LVFX-sensitive strains were detected on day 14.

P. acnes were positive on days −3, 0, and 14, which MIC ranges were 0.5–2, 0.25–1, and 0.5–1 μg/mL, respectively. There were no significant changes in the MIC range due to the LVFX 1.5% administration [$P=0.3739$: day −3 vs. day 0 ($n=5$); $P=0.3910$: day −3 vs. day 14 ($n=4$) using paired t-test].

In all, no microbes were suspected to have acquired drug resistance by the administration of LVFX 1.5%.

TABLE 2. RESULT OF THREE BACTERIOLOGICAL EXAMINATIONS ON DAYS −3, 0, AND 14

Species	*Result on days −3, 0, and 14 (+ −)*					
	+ − −	+ + −	+ − +	+ + +	− + −	− − +
Gram-positive coccus						
MSSA	6/6[a]	—	—	—	—	—
MRSA	2/2	—	—	—	—	—
MSCNS	16/17	—	—	—	—	1/17
MRCNS	24/25	—	1/25	—	—	—
Streptococcus sp	3/3	—	—	—	—	—
E. faecalis	2/3	—	—	—	1/3	—
Corynebacterium sp	40/43	1/43[b]	—	1/43[c]	—	1/43
Gram-negative rods	7/11	—	—	—	—	4/11
P. acnes	14/30	5/30[d]	4/30[e]	—	4/30	3/30

[a]Number of patients applicable to the result per total number of patients with the microbial species.

[b]Microbial substitution was found by the genetic testing for the region of 16s ribosome. (*Corynebacterium macginlery* on day −3, *Corynebacterium striatum* on day 0).

[c]Patient with a complication of nasolacrimal duct obstruction.

[d]No significant change in MIC ($P=0.3739$: day −3 vs. day 0).

[e]No significant change in MIC ($P=0.3910$: day −3 vs. day 14).

—, No patients; +, positive; −, negative; MIC, minimum inhibitory concentration.

TABLE 3. MIC AGAINST LVFX OF GRAM-POSITIVE COCCI ON DAY-3

Species	*Number of patients*	*MIC range (μg/mL)*	*MIC_{50} (μg/mL)*	*MIC_{90} (μg/mL)*
MSSA	6	0.12, 0.25	NC	NC
MRSA	2	0.25, 32	NC	NC
MSCNS	16	0.12–8	0.25	4
MRCNS	25	0.25–32	4	16
Streptococcus sp	2	0.5, NA	NC	NC
E. faecalis	3	1, 2	NC	NC

NC, not calculated; NA, not applicable due to growth insufficiency for antibacterial susceptibility evaluation.

Safety

Among the 96 patients, 4 cases of adverse drug reaction were observed in 4 patients (4.2%). All cases were slight. Superficial punctate keratitis was observed in 3 patients. Two cases, which were observed on days 0 and 8, respectively, disappeared during the LVFX 1.5% administration period after surgery. One case, which was observed on day 14 in the patient with dry eye disease as a complication, recovered soon after day 14. Eyelid swelling was observed in 1 patient on day 0. It disappeared during the LVFX 1.5% administration period after surgery.

Discussion

The preoperative eradication rate by LVFX 1.5% was 86.7% by its administration 3 times a day for 3 days. It was higher compared with 0.5% levofloxacin ophthalmic solution (60.8%).[8] The rate of LVFX 1.5% was higher than those of other fluoroquinolone ophthalmic solutions (70.0%–85.0%),[15,16] even though it is impossible to compare directly the preoperative eradication rate by LVFX 1.5% with those of other fluoroquinolone ophthalmic solutions due to the difference of preoperative dosing regimen.

The preoperative eradication rates were 100% for gram-positive cocci, even including MRSA and MRCNS, which had high MIC against LVFX (32 μg/mL). One of the reasons as to why LVFX 1.5% could eradicate bacteria with such high MIC against LVFX would be the high concentration of LVFX, which could lead to retaining the necessary tissue concentration for exerting antibiotic effects for a long time. In general, the postantibiotic effect (PAE), which indicates a lasting antibiotic effect after the disappearance of the antibiotic drug, is regarded as an important factor for the potency of an antibiotic ophthalmic solution.[27] Although it is reported that the PAE of the fluoroquinolone ophthalmic solution against fluoroquinolone-resistant bacteria was short,[27] the high concentration of LVFX could probably cover the shortness of PAE. It is considered that LVFX 1.5% substantially reduced the concern of postoperative endophthalmitis caused by gram-positive cocci, and further investigation of the mechanism of antibiotic effects and the pharmacokinetics of LVFX 1.5% in the conjunctival sac is necessary.

In contrast, the preoperative eradication rate of *P. acnes* was 78.3%. It was equivalent to those of other fluoroquinolone ophthalmic solutions (76.9%–82.4%).[15,16] Although MIC of LVFX against *P. acnes* isolates are between 0.25 and 2 μg/mL, it was hard to eradicate completely. There are several reasons of ineffectiveness for eradication. Because *P. acnes* is an obligatory anaerobe, *P. acnes* tends to exist where the exposure to air is low, such as in meibomian glands and deep areas of the conjunctival sac. In addition, the generation time of *P. acnes* was reported as 5.1 h, and it was considerably slower compared with *S. aureus* (24 min), which is regarded as a causal microbe for postoperative endophthalmitis.[28] These reasons would reduce the opportunity for *P. acnes* to be eradicated because of DNA gyrase inhibition by fluoroquinolone agents; thus, the preoperative eradication rate of *P. acnes* can be expected to be lower compared with other microbial species. Continuously, further investigation of the perioperative eradication technique of *P. acnes* is considered to be necessary.

There is a concern that low-concentration antibiotic ophthalmic solutions may cause an acquisition of drug resistance. Indeed, it is reported that quinolone-resistant *S. epidermidis* was detected after 21 days of perioperative LVFX 0.5% administration period and there was a gene mutation in the quinolone resistance-determining region of these strains.[18] In this study, there were some strains that were detected on day −3 and not eradicated on days 0 or 14, such as 5 strains of *P. acnes* and 1 strain of *Corynebacterium* on day 0, 1 strain of MRCNS and 4 strains of *P. acnes* on day 14, and 1 strain of *Corynebacterium* on days 0 and 14. This study had a limitation that sufficient evaluation could not be conducted about the influence of LVFX 1.5% on antibacterial susceptibility by perioperative administration, because genetic testing of the quinolone resistance-determining region was not conducted. However, there seemed to be no strains that were suspected to acquire the drug resistance, because apparent increase of MIC in 1 strain of *Corynebacterium* was found to be caused by a microbial substitution during 3 days of administration as a result of a genetic testing for the region of 16s ribosome. Also, there was no apparent increase in MIC days 0 and 14 of these strains from day −3 in other strains.

LVFX 1.5% showed the results of higher preoperative eradication rates and there were no strains suspected to have acquired drug resistance. The reason of the result was considered that LVFX 1.5% contains a high concentration of active antibiotic substance under the consideration of the pharmacokinetic–pharmacodynamic theory.[29] Especially, the higher tissue penetration by instillation of the high-concentration levofloxacin ophthalmic solution may contribute to the result of this study because the levofloxacin solution was shown to penetrate to the bulbar conjunctiva,[21] however, further investigations will be necessary to clarify the mechanism of the antibiotic effect of LVFX in perioperative administration.

The incidence of adverse drug reactions was 4.2%. All cases were slight and disappeared during the administration period after surgery or soon after the end of the administration period. Our data suggest that LVFX 1.5% will have a favorable benefit–risk balance in the clinical use for perioperative disinfection.

Conclusion

For ophthalmic perioperative disinfection, the LVFX 1.5% ophthalmic solution has demonstrated a good safety profile, and overcomes the critical eradication of gram-positive cocci, even including the fluoroquinolone-resistant strains. Further investigation is necessary for the disinfection technique of *P. acnes*.

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Author Disclosure Statement

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Pseudomonas aeruginosa MucD Protease Mediates Keratitis by Inhibiting Neutrophil Recruitment and Promoting Bacterial Survival

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Purpose. *Pseudomonas aeruginosa* is a leading pathogen of blinding keratitis worldwide. In this study, the role of the serine protease in the pathogenesis of *P. aeruginosa* keratitis in the mouse cornea was investigated by comparing the parent and rescue strains.

Methods. Cornea of C57BL/6 mice were infected with *P. aeruginosa* strain PAO1, serine protease (MucD protease or PA3535) mutants (Δ*mucD* or Δ*PA3535*), or a complemented strain. Corneal virulence was evaluated by determining clinical scores and bacterial enumeration. A myeloperoxidase assay was performed to determine the number of polymorphonuclear (PMN) cells infiltrating the cornea. An ELISA was used to quantify inflammatory cytokines and chemokines in the cornea.

Results. The clinical score and bacterial numbers in eyes infected with Δ*mucD* were significantly lower than in those infected with PAO1, Δ*PA3535*, or the MucD rescue strain after 48 hours ($P < 0.001$). A larger number of infiltrating PMN cells was observed in eyes infected with Δ*mucD* at 12 and 24 hours, compared with eyes infected with PAO1. IL-1β, KC, and MIP2 levels were higher in eyes infected with Δ*mucD* than in those infected with PAO1 after 12 hours.

Conclusions. The MucD protease suppressed IL-1β, KC, and MIP2 during the early stages of the infection and inhibited neutrophil recruitment in the cornea. Therefore, the MucD protease contributes significantly to the pathogenesis of keratitis. MucD protease plays a critical role in the establishment of *Pseudomonas aeruginosa* keratitis by facilitating evasion of the immune response.

Keywords: *Pseudomonas aeruginosa*, keratitis, MucD gene, MIP-2, neutrophil

Pseudomonas aeruginosa is a common opportunistic bacterial pathogen that causes a variety of human infections, and is a leading cause of blinding keratitis worldwide. Keratitis caused by *P. aeruginosa* occurs following injury, ocular surgery, or in association with contact lens wear, and can progress rapidly with suppurative infiltration; it can lead to corneal perforation and melt, and result in the loss of vision.[1] To understand the mechanism of pathogenesis in keratitis caused by *P. aeruginosa*, investigators have attempted to identify the virulence factors and their associated cellular structures, such as the flagella,[2,3] pili,[4] and lipopolysaccharide,[5] as well as extracellular products, including proteases,[6–10] exotoxin A,[11] and biofilm.[12] Of these, proteases, including metalloproteases such as alkaline protease, elastase A, and elastase B,[13–15] have been investigated extensively. However, infection of animal models with metalloprotease-deficient mutants demonstrated that none of these enzymes are essential for corneal virulence.[16] Protease IV has been shown to be an important virulence factor in rabbit cornea.[17,18] Moreover, *P. aeruginosa* small protease (PASP), a more recently discovered protease, plays a critical role in the pathogenesis of keratitis.[7,19,20] Along with these virulence factors, the Type III secretion system (TTSS) is involved in the pathogenesis of keratitis.[21–25] The TTSS probes the host cell and transports toxins using a needle-like prong apparatus.

The corneal response to *P. aeruginosa* infection is critical for a better understanding of the natural defense mechanisms of the cornea, which will in turn facilitate the development of novel new treatments and preventive measures.[26] IL-1 and chemotactic cytokines (e.g., IL-8) play a critical role in neutrophil recruitment and the innate immune response.[26,27] Sun et al.[25] showed that ExoS and ExoT ADP ribosyltransferase (ADPR) activities mediated *P. aeruginosa* keratitis in mice by promoting neutrophil apoptosis and bacterial survival. Moreover, ExoS ADPR activities inhibited IL-1β and IL-18 secretion by repressing the activation of caspase-1 in the host cell.[28] Thus, *P. aeruginosa* virulence factors, such as TTSS, may influence the cytokine profile and reduce neutrophil recruitment or activity, promoting bacterial survival in the cornea. Recently, Okuda et al.[29] demonstrated IL-8 degradation following infection of Caco-2 cells with the wild-type, but not the Δ*ExoS*, strain; purified ExoS protein did not degrade IL-8.[29] However, ExoS may degrade IL-8 indirectly; IL-8 degradation by *P. aeruginosa* was blocked by the addition of serine protease inhibitors. Thus, serine proteases may influence the cytokine profile and the

TABLE. Bacterial Strains Used in This Study

Strain	Phenotype	Genotype and/or
PAO1	Prototroph	Wild type
Δ*mucD*	MucD protease-deficient	PAO1 Δ*mucD*
Δ*mucD/mucD*	MucD protease-complement strain	PAO1 Δ*mucD/mucD*, Gm^r
ΔPA3535	PA3535 deficient	PAO1 Δ*PA3535*

Gm^r, gentamicin resistant.

immune response in the cornea. Protease IV, a serine protease, has been shown to be a critical virulence factor in rabbit and mouse corneas,[17,18] and can degrade various proteins, including complement, fibrinogen, plasminogen, immunoglobulin, and surfactant proteins.[30,31] However, little is known of the role of *P. aeruginosa* serine proteases, other than protease IV, in corneal infection. In this study, the role of serine proteases in *P. aeruginosa* keratitis in mice was investigated.

MATERIALS AND METHODS

Animal Care and Use

Female C57BL/6 mice were obtained from CLEA, Japan, Inc. (Tokyo, Japan). Mice at 7 to 9 weeks of age were used in all experiments. All animals were humanely treated according to the guidelines of the ARVO Resolution on the Use of Animals in Research.

Bacterial Strains and Culture Conditions

The wild-type PAO1 strain and mutant strains were used (Table). Searching of the Pseudomonas Genome Database (http://www.pseudomonas.com) revealed two serine protease genes, *PA3535* and *mucD* (*PA0766*).[32] Thus, Δ*mucD* and Δ*PA3535* strains, constructed using the suicide vector pEX18Tc as described previously, were used,[29] as well as the *mucD* (Δ*mucD/mucD*) complemented strain, transformed by a pBBR1MCS5-tac plasmid harboring *mucD*. Bacteria were grown to mid-log phase in brain–heart infusion broth, washed, and diluted in sterile PBS to 1×10^5 cells per 2.5 μL.

In Vivo Model of Corneal Infection

The mouse keratitis model used in this study has been described previously.[27] Mice were anesthetized by intraperitoneal (IP) injection of 0.4 mL 1.2% 2,2,2-tribromoethanol (Sigma-Aldrich Japan, Tokyo, Japan) in saline. Central corneas were scarified with three parallel 1-mm-long abrasions using a 27-gauge needle. A 2.5-μL aliquot containing 1×10^5 colony-forming units (CFUs) of *P. aeruginosa* was applied to the scarified cornea. Sterile PBS was applied to the abraded cornea as a trauma control. A sterile trepan (Biopsy Punch; Kai Medical, Seki, Japan) was used to generate a 2-mm-diameter punch of a silicone hydrogel contact lens (1-DAY ACUVUE True Eye; Johnson & Johnson, New Brunswick, NJ), which was placed over the central cornea to maintain placement of the bacterial suspension.

Scoring of Corneal Opacity

Corneal disease was graded using an established scale: 0: clear or slight opacity, partially or fully covering the pupil; 1: slight opacity, fully covering the anterior segment; 2: dense opacity, partially or fully covering the pupil; 3: dense opacity, covering the entire anterior segment; and 4: corneal perforation or phthisis.[33] A clinical score was recorded for each mouse after infection for statistical comparison of disease severity.

Quantification of Bacterial Growth in the Cornea

Whole eyes or corneas were homogenized twice under sterile conditions using the Micro Smash MS-100 system (Tomy Seiko, Tokyo, Japan) at 825*g* for 1 minute. Serial log dilutions were performed and bacteria were plated onto brain–heart infusion agar (BD Biosciences, Franklin Lakes, NJ). Plates were incubated at 37°C for 18 hours, and the number of CFUs was determined by direct counting.

Histology and Immunohistochemistry

Eyes were enucleated at predetermined time points and fixed in 4% paraformaldehyde or methanol overnight at 4°C. They were then embedded in paraffin, and 5-μm sections were cut through the central cornea and stained with hematoxylin and eosin. Rabbit anti-mouse polymorphonuclear (PMN) antibody (1:1000 dilution; Cedarlane Laboratories, Ontario, Canada) was used. Fluorescein isothiocyanate–labeled anti-rabbit IgG (1:500 dilution; Vector Laboratories, Burlingame, CA) was used as the second antibody. The cornea sections were examined and photographed with a charge-coupled device (CCD) camera (model DP-50; Olympus, Tokyo, Japan) attached to a model BX-50 microscope (Olympus) or an inverted fluorescence microscope (Observer Z1; Carl Zeiss Micro Imaging, Thornwood, NY).

Myeloperoxidase Assay

A myeloperoxidase (MPO) assay was modified and used to enumerate active PMN cells that infiltrated the corneal stroma after infection.[34] A 150-μL aliquot, containing homogenized corneas in PBS, was added to 150 μL 100 mM phosphate buffer (pH: 6.0) containing 0.5% hexadecyltrimethylammonium bromide. Samples were freeze-thawed three times and centrifuged at 10,000*g* for 15 minutes at 4°C. Then, a 20-μL aliquot of the supernatant was added to 80 μL of a 50-mM phosphate buffer containing ortho-dianisidine dihydrochloride (16.7 mg per 100 mL) and 0.0005% hydrogen peroxide. The change in absorbance at 450 nm was measured continuously for 5 minutes, and the rate of change for each sample was determined. MPO/cornea units were calculated from a standard curve generated with purified MPO (product number M6908; Sigma-Aldrich Japan). One unit of MPO activity was equivalent to approximately 2×10^5 PMN cells mL^{-1}.

ELISA

Cytokine protein levels were determined using ELISA kits (R&D Systems, Minneapolis, MN). Corneas from mice were collected individually ($n = 5$–6 per group per time point). The corneas were homogenized in 0.5 mL PBS. All samples were centrifuged at 15,500*g* for 5 minutes, and an aliquot of each supernatant was assayed in duplicate for IL-1β, TNF-α, keratinocyte-derived cytokine (KC), and macrophage inflammatory protein-2 (MIP-2) protein, according to the manufacturer's instructions.

Neutrophil Depletion Mice

Mice were rendered neutropenic by IP injection of 100 μg anti-mouse Gr-1 (RB6-8C5 MAb; R&D Systems) in 0.2-mL PBS at 24