(7/47), respectively. The prevalence of *E. chattoni* and *E. hartmanni* examined microscopically and by PCR was 17% (8/47) and 11% (5/47), respectively (Table 2); their fragments were confirmed by nucleotide sequencing and corresponded to the sequence dates of *E. hartmanni* (GenBank accession no. AF149907) and *E. chattoni* (GenBank accession no. AF149912). The amplified PCR products are shown in Figure 2.

Polymorphic genes in JSK04-Eh-V isolates

Genotyping based on the nucleotide sequencing of the chitinase, SREHP, and locus 1-2 genes was applied to the genotyping of JSK04-Eh-V. The DNA samples of JSK04-Eh-V from each of the six primates were subjected to PCR to detect the fragments of the chitinase, SREHP, and locus 1-2 genes. The PCR products of the chitinase genes from the DNA samples of two primates and JSK2004cl2 and the PCR products of the locus 1-2 genes from the DNA samples of four primates and JSK2004cl2 were sequenced; however, the PCR products of the SREHP genes were obtained only from the DNA sample of JSK2004cl2. There was perfect homology between the sequences of the PCR products of the chitinase genes obtained from two primates (one Geoffroy's spider monkey and one white-faced saki) and the PCR products of the locus 1-2 genes from four primates (two Abyssinian colobus monkeys, one Geoffroy's spider monkey, and one white-faced saki). Moreover, the sequences of the two genes of JSK2004cl2 (locus 1-2: GenBank accession no. AB426704; chitinase: GenBank accession no. AB426705) also demonstrated perfect homology. However, the sequence data of the chitinase and SREHP genes of the other two types of *E. histolytica*–like variants (GenBank accession nos. AB282755 and AB197935) isolated from the cynomolgus and rhesus monkeys were different compared to the sequence data of the chitinase gene and SREHP gene (GenBank accession no. AB426706) of JSK2004cl2 (Figs. 3, 4).

Growth kinetics of amoebae in YIMDHA-S

The growth kinetics of axenically grown *E. histolytica* (HM-1:IMSScl6), *E. dispar* (AS 16 IR and CYNO 09:TPC), and JSK04-Eh-V (JSK2004) in YIMDHA-S are shown in Figure 5. These established axenic strains adapted to the YIMDHA-S culture conditions within three subcultures; thereafter, they were inoculated into the YIMDHA-S from the classic TYI-S-33[3] (HM-1:IMSScl6) or YIGADHA-S (AS 16 IR and CYNO 09:TPC) media.

DISCUSSION

The multiplex PCR for *E. histolytica*, JSK04-Eh-V, and *E. dispar* permits species identification in a single reaction mixture and is, therefore, more cost effective and useful for prevention of contamination of DNA samples.

Surveillance of the prevalence of JSK04-Eh-V infection among the primates in the zoo was conducted using multiplex PCR for differential diagnosis of *E. histolytica*, JSK04-Eh-V, and *E. dispar*. Multiplex PCR was confirmed as a useful method for the detection and identification of *E. histolytica*, JSK04-Eh-V, and *E. dispar* in nonhuman primates and even in the zookeepers who were in contact with the primates, because the specificity and reproducibility of this technique were adequate for efficient surveillance of JSK04-Eh-V in the present study.

Concerning the microscopic stool examination process in this survey, amoebic cysts or trophozoites were not always detected in every stool sample obtained from individuals infected with JSK04-Eh-V and *E. dispar*. These cysts or trophozoites could be detected only in one third to two thirds of the stool samples, despite the collection of samples from each individual primate once a day for 3 days. The results indicated that performing a stool examination per day (at least three times) on alternate days is necessary.

The JSK04-Eh-V strain of *E. histolytica* was detected by using the *E. histolytica II* kit, an *E. histolytica*–specific antigen (adhesin) detection kit. It is reported that one of the factors determining the pathogenicity of *E. histolytica* is the cytolysis of host cells that begins with the adhesion of the amoebae to the mucosal target cells of the large intestine via galactose/*N*-acetyl D-galactosamine-inhibitable (Gal/GalNAc) lectin.[8,9] The detection of the *E. histolytica*–specific antigen from JSK04-Eh-V by using the *E. histolytica II* kit indicated that the Gal/GalNAc lectin structure in JSK04-Eh-V is identical to that in *E. histolytica*.

Although the nucleotide sequence of the polymorphic SREHP gene from five primates, except for JSK2004cl2, could not be amplified by PCR, the polymorphic chitinase and locus 1-2 gene sequences from three and six primates, respectively, were observed to be identical. The reasons for the inability of PCR to amplify the SREHP gene were thought to be related to the small amount of JSK04-Eh-V DNA in the stool and liver abscess samples, which were insufficient for the PCR, and the presence of a few irrelevant PCR fragments in each case. Therefore, JSK04-Eh-V infections that oc-

curred in the zoo was presumed to have been spread by a single strain, because the infection was limited to primate groups within a particular zone of the zoo at around the same time. The route of transmission of the infection from the isolated group of primates in captivity, including individuals infected with JSK04-Eh-V, to the other groups has not been determined. It is possible that the cysts are the causative agents of JSK04-Eh-V infection, because *E. histolytica* cysts have been reported to be capable of surviving and retaining their infectivity for a month under appropriate wet conditions.[17]

The symptoms of the zoo primates infected with JSK04-Eh-V differed considerably depending on their species; the symptoms in the De Brazza's guenon were relatively mild, while symptoms in the Abyssinian colobus monkey and Geoffroy's spider monkey were severe and fatal. There appear to be species-specific differences among the primates with regard to susceptibility. Although the transmission route was not clear, it is possible that the primates may be carriers and may thus be a source of the parasite. Prior to this study, JSK04-Eh-V infection was thought to have been eradicated, owing to the diligence of the veterinarians and zookeepers working in the zoo, and, fortunately, no zoonotic infection (including amoebiasis) was found among the zookeepers.

YIMDHA-S was designed for the axenic culture of *E. histolytica*, JSK04-Eh-V, and *E. dispar*. This medium is considered to be efficient in comparing biological characteristics of JSK04-Eh-V with *E. histolytica* and *E. dispar*, such as the intensity of in vitro virulence to mammalian tissue culture cell lines,[9] in a single medium under the same culture conditions.

Acknowledgment: A part of this work was supported by a Health Sciences Research Grant-in-Aid for Emerging and Reemerging Infectious Diseases from the Ministry of Health, Labour and Welfare of Japan.

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Received for publication 12 December 2007

Jpn J Ophthalmol 2008;52:24–31
DOI 10.1007/s10384-007-0485-7

CLINICAL INVESTIGATION

Clinical Application of Real-time Polymerase Chain Reaction for Diagnosis of Herpetic Diseases of the Anterior Segment of the Eye

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Abstract

Purpose: To assess the value of quantification of herpes simplex virus (HSV) DNA for the differential diagnosis of herpetic diseases of the anterior segment of the eye.

Methods: One hundred forty-four samples from 90 patients with ocular inflammatory diseases were examined for HSV DNA by real-time polymerase chain reaction (PCR) with primers set for the consensus sequence of HSV-1/2 DNA polymerase. The samples included corneal epithelial scrapings, tear fluid (200μl of eye wash), and aqueous humor.

Results: In cases of typical herpetic epithelial keratitis, a large number of copies of HSV DNA were detected (mean, 1.0×10^7 copies in epithelial scrapings and 3.5×10^5 copies in tear fluid). In atypical epithelial keratitis cases, a smaller number of HSV DNA copies were detected. In stromal keratitis cases, the number of copies of HSV DNA in the tear fluid (mean: 4.7×10^2 copies) was significantly smaller than in cases of epithelial keratitis. In the aqueous humor, the number of copies was small in endotheliitis cases (mean, 2.9×10^2 copies/μl), but the range was great, from $(1.2–4.8) \times 10^5$/μl in herpetic iridocyclitis. Seventeen percent of cases in which HSV was not suspected to be involved showed a small number of copies of HSV DNA, indicating the unexpected involvement of HSV in these cases.

Conclusions: Real-time PCR is an informative method of diagnosing herpetic eye diseases and evaluating the possible involvement of HSV in other inflammatory ocular diseases. **Jpn J Ophthalmol** 2008;52:24–31 © Japanese Ophthalmological Society 2008

Key Words: aqueous humor, corneal epithelial scrapings, herpes simplex virus, real-time polymerase chain reaction, tear fluid

Introduction

Herpes simplex virus (HSV) keratitis is still a diagnostic and therapeutic challenge to ophthalmologists in spite of the availability of advanced diagnostic methods and the effectiveness of antiviral drugs such as acyclovir. HSV is a latent infection in the body, mainly in the trigeminal ganglia, and can be reactivated by fever, trauma, stress, immunosuppressive agents, or exposure to ultraviolet radiation. In recurrent attacks, the virus is transported anterogradely by the axoplasmic flow in neurons and replicates within corneal epithelial cells. Because of recurrent episodes and the consequent immune reaction in the corneal stroma, severe visual impairment can develop, often necessitating keratoplasty.

Isolation of the virus from tissue is the standard procedure for diagnosing HSV infections, and in 2002, the Ocular Herpetic Infection Research Group stated that a definitive diagnosis of herpetic epithelial keratitis can be made only by viral isolation.[1] However, the isolation of the virus is time-consuming, has poor sensitivity, and requires a special laboratory for virological processing.

Received: January 29, 2007 / Accepted: July 30, 2007
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Another diagnostic test is immunofluorescent staining, which is as valuable for diagnosis as the clinical observation of the typical dendritic keratitis.[1] However, false positive and false negative results are possible, especially in the hands of unskilled examiners.

Polymerase chain reaction (PCR) can also be used to detect HSV DNA. This test is very sensitive and has a relatively rapid processing time.[2] However, at present, PCR is considered only an auxiliary diagnostic method, because its high sensitivity also detects nonpathogenic viruses shed spontaneously from the trigeminal ganglia. Although, theoretically, high sensitivity can be attained with PCR, its actual sensitivity depends on the testing conditions, such as the primers used; therefore, PCR results from different laboratories cannot be directly compared.

Herpetic iridocyclitis is a poorly diagnosed disease because it does not have specific clinical features, and diagnostic methods to identify the causative agent are not available in the clinic. However, it was recently reported that PCR can be used to diagnose herpetic iridocyclitis,[3] although its clinical value is still uncertain, for the same reasons as with herpetic keratitis, namely, it also detects nonpathogenic viruses shed spontaneously from the trigeminal ganglia.

To compensate for this drawback of PCR and to establish its diagnostic value in herpetic eye diseases of the anterior segment, we applied real-time PCR, which enabled us to quantify the viral DNA. We assessed the clinical value and efficacy of HSV DNA quantification for differential diagnosis of herpetic keratitis and iridocyclitis to determine the threshold amount of HSV DNA necessary for the proper diagnosis of herpetic eye diseases.

Subjects and Methods

Patients

Ninety patients with clinically diagnosed herpetic ocular diseases or other inflammatory ocular diseases of the anterior segment who were treated at the Tottori University Hospital between July 2003 and December 2005 were enrolled in this study. Forty-eight of the patients were male, and 42 were female, and their ages ranged from 10 months to 88 years, with a mean of 56.3 years.

The types of herpetic eye diseases in the anterior segment in these patients were divided into six categories by using the following criteria:

1. Typical epithelial keratitis: dendritic or geographic epithelial defects with terminal bulbs and swelling or infiltration of the epithelial edge.
2. Atypical epithelial keratitis: dendritic or geographic epithelial defects but without terminal bulbs and swelling or infiltration of the epithelial edge, and without any obvious cause other than HSV.
3. Typical stromal keratitis: disciform keratitis (appearing as round opacities or edema with keratic precipitates), or necrotizing keratitis (appearing as dense infiltrations with stromal neovascularization), with corneal hypesthesia and a past history of herpetic keratitis.
4. Atypical stromal keratitis: stromal edema or infiltration, without a past history of herpetic keratitis or corneal hypesthesia, and without any cause other than HSV.
5. Iridocyclitis: unilateral iridocyclitis with recurrence and poor response to steroid therapy.
6. Endotheliitis: focal corneal edema with keratic precipitates and a small number of cells in the anterior chamber, and without obvious stromal or epithelial involvement.[4]

All patients signed an informed consent from after an explanation of the purpose of the study and the procedures to be used. The procedures used conformed to the tenets of the Declaration of Helsinki and were approved by the Institutional Review Board of Tottori University.

Sample Collection

Three types of samples were collected: (1) Tear fluid was collected as eyewash by rinsing the ocular surface with 500 μl of sterile saline three times, and 200 μl of the 500 μl collected from each subject was used for DNA extraction. (2) Epithelial scrapings were collected by debridement of the edge of an ulcer with MQA tips. (3) Fifty microliters of aqueous humor was collected by anterior chamber paracentesis with a 27-gauge needle.

We collected a total of 144 samples from the participants in this study. We collected 15 samples from eight patients with typical epithelial keratitis, 13 samples from six patients with atypical epithelial keratitis, ten samples from seven patients with typical stromal keratitis, 18 samples from 13 patients with atypical stromal keratitis, eight samples from five patients with endotheliitis, and seven samples from four patients with iridocyclitis. We also tested 73 samples from 47 patients with other inflammatory diseases of the anterior segment in which herpes was not suspected. In addition, we collected 24 samples of tear fluid from 12 individuals without any ocular inflammatory diseases as normal controls.

After collecting the samples, we carefully documented the clinical course of the disease, especially the efficacy of antiviral therapy with acyclovir and valacyclovir.

Real-Time Polymerase Chain Reaction

The real-time PCR assay was carried out with a QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) with primers specific to the DNA polymerase region of HSV type 1 (HSV-1) and HSV type 2 (HSV-2) (92 base pairs). The forward primer sequence was 5′-CAT CAC CGA CCC GGA GAG GGA C-3′, and the reverse primer sequence was 5′-GGG CCA GGC GCT TGT TGG TGT A-3′. DNA was extracted from 200 μl of eye wash. Aqueous humor was diluted to a 200-μl volume with phosphate-buffered saline.

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Debrided epithelial samples were prepared by using a DNAQIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the standard protocol, and eluted in 50μl of distilled water. Eight microliters of the DNA extracted from each sample was used for the real-time PCR assay.

Following one cycle of 15min at 95°C, 45 three-step cycles of 15s at 94°C (denaturation step), 20s at 56°C (annealing step), and 10s at 72°C (extension step) were carried out in a LightCycler (Roche Diagnostic, Nutley, NJ, USA). The PCR-amplified DNA polymerase region of HSV was cloned using pT7Blue T vector-2 (Novagen, Darmstadt, Germany). Serial dilution of the restriction enzyme-digested and cloned template was used to generate a standard curve. Crossing points of real-time fluorescence were normalized to calculate copy numbers of the HSV transcript in the samples according to the standard curve with LightCycler Software, Version 3.5 (Roche Diagnostic) (Fig. 1).

To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and subsequent agarose gel electrophoresis. The identity of the amplified product was further confirmed by using PCR-based direct sequencing.

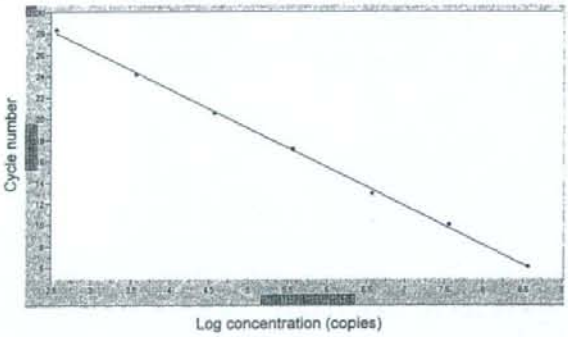

Figure 1. Standard curve of herpes simplex virus (HSV) transcripts. The PCR-amplified DNA polymerase region of HSV was cloned using pT7B1ue T-vector-2 (Novagen). Serial dilution of the restriction enzyme-digested and cloned template was used to generate a standard curve.

Results

The percentage of specimens confirmed to have HSV DNA by real-time PCR in each of the six disease categories is shown in Table 1. The highest positive percentage was obtained in samples of typical epithelial keratitis ($n = 15$; 100%), and the lowest percentage was found in samples from endotheliitis cases ($n = 8$; 25%). The positive percentage was lower in typical and atypical stromal keratitis cases than in either typical or atypical epithelial keratitis cases. Even among cases of anterior segment inflammation tested to exclude herpes, 12.3% of samples were positive and 17.0% of cases.

The number of copies of HSV DNA in typical epithelial keratitis patients was $>10^5$ copies/whole collected tissue (mean, 1.0×10^7 copies/sample) in epithelial scrapings, and $>10^4$ copies (mean, 3.5×10^5 copies/sample) in 200μl of eye wash (tear fluid). The number of copies of HSV DNA was significantly smaller in tear fluid than in epithelial scrapings (Student t test, $P < 0.05$) (Fig. 2). Acyclovir ointment was effective in all of these cases.

In cases of typical stromal keratitis, 5/10 (50.0%) of samples and 5/7 (71.4%) of cases were positive for HSV DNA. In positive cases, the number of copies of HSV DNA ranged from 10^1 to 10^3 copies (mean, 4.7×10^2 copies/sample). The number of copies of HSV DNA in tear fluid was significantly smaller in cases of typical stromal keratitis than in cases of typical epithelial keratitis (Student t test, $P < 0.05$) (Fig. 2). Epithelial scrapings were collected from only one patient with necrotizing keratitis, and 2.8×10^5 copies of HSV DNA were detected. This patient was treated successfully with topical steroid and antiviral drugs.

In cases of atypical epithelial keratitis (Table 2), 10^2 to 10^3 copies of HSV DNA were detected in both tear fluid and epithelial scrapings, except in three samples from two cases in which 10^5 copies were detected (mean, 3.7×10^4 copies in epithelial scrapings, 1.3×10^3 copies in tear fluid). The number of copies of HSV DNA in tear fluid in cases of atypical epithelial keratitis was significantly smaller than that in typical epithelial keratitis cases (Student t test, $P < 0.05$). Antiviral drugs were effective in all positive cases. In one case that was resistant to acyclovir (case 2), 1% trifluorothymidine was effective. Two patients who were negative by real-time PCR (cases 5 and 6) were later diagnosed as

Table 1. Cases positive for herpes simplex virus (HSV) DNA in each type of disease in real-time polymerase chain reaction findings

Category	Positive samples (%)	Positive cases (%)
Typical epithelial keratitis	15/15 (100)	8/8 (100)
Atypical epithelial keratitis	10/13 (76.9)	4/6 (66.7)
Typical stromal keratitis	5/10 (50.0)	5/7 (71.4)
Atypical stromal keratitis	7/18 (38.9)	7/13 (53.8)
Endotheliitis	2/8 (25.0)	1/5 (20.0)
Iridocyclitis	6/7 (85.7)	3/4 (75.0)
HSV not suspected	9/73 (12.3)	8/47 (17.0)
Total	144 samples	90 cases

Table 2. Cases of atypical epithelial keratitis

Case no.	Age (years)	Sex	Clinical characteristics	Sample type	Copy number[a]
1	50	F	Fine dendrite after trabeculectomy	Debridement	3.0×10^2
2[b]	87	F	Partial dendritic lesion	Tear	1.9×10^2
			Partial dendritic lesion	Tear	1.5×10^2
	88		Partial dendritic lesion	Tear	1.1×10^3
			Partial dendritic lesion	Tear	7.5×10^2
			Partial dendritic lesion	Tear	7.6×10^2
3	77	M	Dendritic lesion adjacent to pterygium head	Debridement	7.7×10^5
				Tear	5.0×10^5
4	24	M	Dendritic lesion on peripheral cornea	Tear	8.6×10^2
				Debridement	2.2×10^5
5	72	M	Dendritic lesion on band keratopathy	Tear	(–)
6	78	M	Dendritic lesion on peripheral cornea	Tear	(–)
				Debridement	(–)

[a]Number of copies in 200 μl in tears or tissue collected during debridement.
[b]Five tear samples were collected at different times.

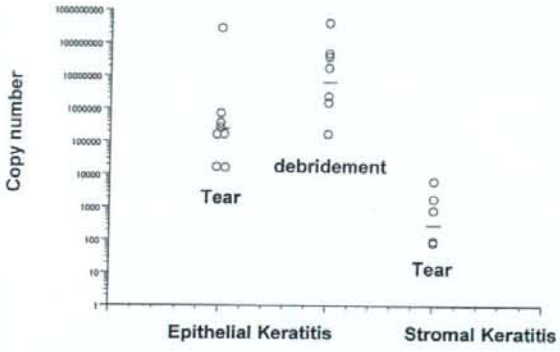

Figure 2. Copy numbers of HSV-DNA in typical epithelial and stromal keratitis. In cases of typical epithelial keratitis, HSV-DNA was detected with more than 105 copies (mean, 1.0×107 copies/sample) in epithelial scrapings and more than 104 copies (mean, 3.5×105 copies/sample) in tear fluid (200 μl of eye wash). The number of copies of HSV-DNA in the tear fluid was significantly lower than in the epithelial scrapings (Student *t* test, $P < 0.05$). HSV-DNA in tear fluid of typical stromal keratitis cases was significantly less than that of typical epithelial keratitis cases (Student *t* text, $P < 0.05$). Each *point* indicates the copy number of each sample. Each *bar* indicates the mean copy numbers. O, sample; —, mean.

not having herpetic keratitis, because they were not responsive to acyclovir. The epithelial defect in case 5 was later identified as a pseudodendrite related to band keratopathy, and the epithelial defect in case 6 was identified as a catarrhal ulcer.

In cases of atypical stromal keratitis (Table 3), 7/18 (38.9%) samples and 7/13 (53.8%) patients were positive for HSV DNA. In the positive cases, 10^1 to 10^3 copies of HSV DNA were detected in both tear fluid and aqueous humor (mean, 9.5×10^2 copies in tear fluid). All of these cases were successfully treated with steroid and antiviral drugs.

Among the patients diagnosed with endotheliitis ($n = 5$; Table 4), 1.5×10^2 and 4.3×10^2 copies/μl HSV DNA were detected in two samples of aqueous humor. This patient (case 1) was a 59-year-old man who had had keratoplasty because of herpetic keratitis and corneal edema in which keratic precipitates involving the host cornea had developed in September 2003 and March 2005. The signs of endotheliitis disappeared after administration of valacyclovir on both occasions.

Four cases of iridocyclitis were examined because HSV was suspected as a causative agent (Table 5), and HSV DNA was detected in the aqueous humor with copy numbers ranging from 1.2×10^1 to 4.8×10^5/μl in three of the cases. Case 1 was successfully treated with steroid and antiviral drugs, and in case 3, a gradual decrease in the number of copies of HSV DNA and a reduction of aqueous inflammation were observed after steroid and acyclovir ointment with oral valacyclovir. These therapies were not effective for case 2, in which a small number of copies of HSV DNA was observed.

Among cases of anterior segment inflammatory diseases examined in which herpetic disease was not suspected, eight of the 47 cases (17.0%) demonstrated HSV DNA. The cases positive for HSV DNA are summarized in Table 6. The copy numbers in these cases ranged from 10^2 to 10^3 in tear fluid (200 μl of eye wash) and 10^3 copies in epithelial scrapings. In five of the eight positive cases, antiviral treatment was applied, and two (cases 3 and 6) were responsive to this treatment. HSV DNA-negative cases included four cases of catarrhal ulcer, four of bacterial keratitis, three of varicella-zoster virus (VZV) keratitis, three of rejection, three of Stevens-Johnson syndrome.

In the normal control eyes, HSV DNA was not detected. The number of copies in each disease category is summarized in Table 7.

Discussion

PCR is widely used in the clinic and in the laboratory, and new methods have been developed such as nested PCR,[5] multiplex PCR,[6] and competitive PCR.[7] Although real-time

Table 3. Cases of atypical stromal keratitis

Case no.	Age (years)	Sex	Clinical diagnosis and characteristics	Sample	Copy number[a]
1	4	M	Patchy infiltrations and superficial punctate keratopathy	Tear	1.2×10^2
2	26	M	Peripheral corneal infiltrates and limbitis	Tear	4.0×10
3	33	M	Deep stromal keratitis	Tear	2.0×10^3
4	73	M	Necrotizing keratitis + trophic ulcer	Tear	1.5×10^3
5	68	M	Corneal anterior stromal opacity (without edema or keratic precipitates)	Tear	1.0×10^3
6	70	M	Scarring opacity	Tear	9.4×10^2
7	68	F	Keratouveitis (slight stromal opacity)	Aqueous humor	1.2×10^2
				Tear	(–)
8	72	F	Stromal keratitis + suspected toxicity of eye drops	Tear	(–)
				Debridement	(–)
9	63	M	Keratouveitis + suspected bacterial keratitis	Tear	(–)
				Debridement	(–)
10	64	F	Stromal keratitis (with atypical dendrite)	Tear	(–)
11	37	M	Stromal keratitis (without corneal hypesthesia)	Tear	(–)
			Stromal keratitis (without corneal hypesthesia)	Tear	(–)
12	71	F	Stromal keratitis (severe corneal opacity)	Tear	(–)
				Debridement	(–)
13	28	M	Multiple subepithelial infiltrates on graft	Tear	(–)

[a]Copy number/200μl in tears or tissue collected during debridement, or in 1μl of aqueous humor.

Table 4. Cases of endotheliitis

Case no.	Age (years)	Sex	Sample	Response to antiviral drugs	Copy number[a]
1[b]	59	M	Aqueous humor	Good	1.5×10^2
	60	M	Aqueous humor	Good	4.3×10^2
2[c]	59	F	Aqueous humor	Good (with steroid)	(–)
3	72	F	Aqueous humor	Not used	(–)
			Tear	Not used	(–)
4	71	M	Aqueous humor	Good	(–)
	72	M	Aqueous humor	Not obvious	(–)
5	54	M	Aqueous humor	Good	(–)

[a]Copy number/μl aqueous humor.
[b]Positive for varicella-zoster virus DNA.
[c]Two aqueous humor samples were collected at different times.

Table 5. Cases of iridocyclitis suspected to be herpetic infections

Case no.	Age	Sex	Sample	Response to antiviral drugs	Copy number[a]
1	55	F	Aqueous humor	Good	1.6×10^3
2	65	F	Aqueous humor	Not obvious	2.1×10
3[b]	57	F	Aqueous humor	Relatively effective with steroid	4.8×10^5
			Aqueous humor	Relatively effective with steroid	5.1×10^4
			Aqueous humor	Relatively effective with steroid	1.2×10^4
	58	F	Aqueous humor	Relatively effective with steroid	1.2×10
4	46	M	Aqueous humor	Not used	(–)

[a]Copy number/μl of aqueous humor.
[b]Four aqueous humor samples were collected at different times.

PCR requires special equipment, for example, a LightCycler, which is the combination of a thermal cycler and a spectrofluorophotometer, the results are reproducible and the technique is very sensitive. In addition, the methods are simple and relatively quick because electrophoresis is not necessary. More importantly, this technique can quantify the amount of DNA accurately in the range of exponential amplification. Thus, real-time PCR should be very helpful for diagnosing herpetic diseases, which are difficult to diagnose merely by the presence of viral DNA because of latent infections and the possibility of viral shedding. However, only a few reports have been published on the clinical application of real-time PCR to herpetic keratitis[8–10] and iridocyclitis, and only one report summarizes a large number of cases.[11] Our current study was done to provide more information on the relation between clinical manifestations and

Table 6. HSV DNA-positive cases among those in which herpetic infection was not suspected

Case no.	Age (years)	Sex	Clinical characteristics & diagnosis	Sample	Copy number[a]	Evidence of other causes
1[b]	58	M	*Acanthamoeba* keratitis	Debridement	1.0×10^3	Cysts (+) in smear
2[b]	56	F	Bullous keratopathy	Aqueous humor	7.7×10	(–)
3[b]	64	M	Epithelial defect in Stevens-Johnson syndrome	Tear	3.8×10^3	(–)
4	68	M	Fungal keratitis in patient with graft-versus-host disease	Debridement	2.8×10^3	Hyphae (+) in smear
5	50	F	Persistent epithelial defect after PKP	Tear	1.0×10^3	(–)
6[b]	37	F	Iridocyclitis and limbitis after subconjunctival tumor resection	Tear	8.5×10^2	(–)
7[b]	85	F	Fungal keratitis	Tear	2.0×10^3	Culture for fungi (+)
				Debridement	2.4×10^3	Culture for fungi (+)
8	60	F	Peripheral corneal ulcer	Tear	1.3×10^3	(–)

PKP, penetrating keratoplasty.
[a]Copy number/200 µl in tears or in tissue collected during debridement, or in 1 ml aqueous humor.
[b]Antiviral treatment was applied depending on the results of real-time polymerase chain reaction.

Table 7. Summary of copy numbers in each type of disease

Disease type	Sample type	Copy number
Epithelial (typical)	Tear	10^4–10^8
	Debridement	10^5–10^8
Epithelial (atypical)	Tear · debridement	10^2–10^3 (except 3 samples at the 10^5 level)
Stromal (typical)	Tear	10^1–10^5
Stromal (atypical)	Tear	10^1–10^3
Endotheliitis	Aqueous humor	10^2
Iridocyclitis	Aqueous humor	10^1–10^5
Herpetic infection not suspected	Tear	10^2–10^3
	Debridement	10^3

viral amounts, and to establish a basis for diagnosis of herpetic keratitis and iridocyclitis by real-time PCR. Because we selected primer sets that were the consensus sequence of HSV-1/2 DNA polymerase, it was not possible to determine whether the detected DNA was from HSV-1 or HSV-2. It is, however, highly likely that the detected DNA belongs to HSV-1 because of the rarity of HSV-2 as a causative agent of herpetic keratitis and iridocyclitis.[12]

In typical herpetic epithelial keratitis, more than 10^5 copies were detected in epithelial scrapings and more than 10^4 copies in tear fluid. This level of copy number was never found in cases of other ocular anterior segment inflammatory diseases. Therefore, these data suggest that when the copy number is higher than 10^4 in the tear fluid, a definite diagnosis of herpetic keratitis can be made.

In atypical epithelial keratitis, a few cases with high copy numbers of $>10^4$ were found, but usually the copy number was low, suggesting that the atypical property of this epithelial keratitis was most likely due to lower proliferation of the virus. The all cases which we diagnosed atypical epithelial keratitis responded to antiviral drugs and HSV DNA was not detected after healing in some cases. So we considered all of the positive cases to be herpetic in origin. Also in the cases that were negative by real-time PCR, acyclovir was not effective. This consistency between real-time PCR results and responsiveness to antiviral drugs supports the usefulness of real-time PCR. Although a positive result alone cannot definitely indicate HSV as the causative agent, we can eliminate HSV as the cause of epithelial keratitis by negative results of real-time PCR.

In both typical and atypical stromal keratitis patients, the percentage of positive HSV DNA specimens and the number of copies of HSV DNA were smaller than in epithelial keratitis patients. A smaller number of positive percentages in stromal keratitis cases was also found by PCR (2/6, 33.3%),[2] nested PCR (5/15, 33.3%),[5] and real-time PCR (8/14, 57.1%).[11] These data are comparable to ours: 50.0% of samples and 71.4% of patients with typical stromal keratitis, and 38.9% of samples and 53.8% of patients with atypical stromal keratitis. Fukuda et al.[11] found no significant differences in levels of HSV DNA between eyes with epithelial keratitis and those with stromal keratitis. Considering the low viral isolation rate[1] and the absence of active replicating HSV on the ocular surface in stromal keratitis, our data are reasonable and easily explainable. Fukuda et al.[11] divided cases of stromal keratitis into active disciform stromal keratitis and silent stromal keratitis, and obtained high copy numbers in the active type but not in the silent type. Therefore, the inclusion of several cases of the very active-type stromal keratitis and the exclusion of the silent

type probably raised the copy numbers in their study. In fact, one of our cases with epithelial involvement had high copy numbers in epithelial scrapings.

We detected HSV DNA in only one of our cases of endotheliitis, and the copy number was low. Even in the other patients with good response to antiviral drugs, HSV DNA was not detected. Only one study has reported isolating HSV DNA in endotheliitis,[13] and none was detected in the three cases of endotheliitis reported by Fukuda et al.[11] Considering these findings together with our results, we conclude that the proliferation of HSV is very low in cases of endotheliitis. Because human endothelial cells do not proliferate in vivo, the low levels of HSV DNA in herpetic endotheliitis are reasonable, because the virus can proliferate in dividing cells but not in nondividing cells.

One other possible explanation for the low percentage of HSV DNA detected in endotheliitis is that some of the cases were caused by viruses other than HSV. In fact, one of our cases (case 3 in Table 4) demonstrated a low copy number of VZV DNA by real-time PCR (data not shown) indicating that the causative agent was VZV. Recently, endotheliitis due to cytomegalovirus (CMV) has been reported.[14] Cases of CMV endotheliitis were probably included in our samples. However, we do not know exactly how many were present, because we did not perform real-time PCR for CMV.

In cases of iridocyclitis, varying numbers of copies were detected. In the patient with the highest copy number of HSV DNA, the copy number did not decrease for a long time, in spite of good clinical response to antiviral drugs and steroids. In some cases of acute retinal necrosis, a slow decrease of viral DNA has been reported.[15,16] Probably, more time is required to eliminate viral DNA in the closed environment of intraocular inflammation than in the open environment of the ocular surface in herpetic keratitis.

Although HSV DNA was not detected in normal control eyes, 17% of the cases of anterior segment inflammatory diseases examined to exclude HSV were positive for HSV DNA. However, fewer than 10^4 copies of HSV DNA were detected in tear fluid in these cases, and the typical appearance of herpetic keratitis was not present. In two cases, secondary participation of HSV was assumed because of the response to additional use of antiviral drugs. In other cases, the contribution of HSV was not obvious; however, we could not completely eliminate HSV as the causative pathogen.

Cantin et al.[17] have reported that four of 11 corneas from subjects with no history of herpetic eye disease showed the presence of HSV DNA by PCR. Also Garweg et al.[18] reported that 16 of 72 corneal buttons obtained during keratoplasty in cases of nonherpetic keratitis had HSV DNA confirmed by PCR. Moreover, Crouse et al.[19] detected HSV DNA in 3.1% of normal corneal epithelium specimens obtained from cadavers, and Openshaw et al.[20] found that ten of 24 normal donor corneas had HSV DNA when tested by PCR. These results together with reports of the transmission of HSV by donor corneas[21–23] indicate the ubiquitous nature and unexpected presence of HSV.

On the basis of these findings, we speculate that severe inflammation or extensive use of steroids may have activated latent HSV in some of our patients with ocular anterior segment inflammatory diseases. We assume that the reactivated virus was not the main cause of inflammation in these cases, but exacerbated the inflammation or induced additional, secondary lesions. Indeed, Kaufman et al.[24] have reported that the use of conjunctival swabs merely twice daily for 30 days, which is a minor insult to the ocular surface, induced a high frequency (up to 98%) of spontaneous shedding. Considering their results, our speculation is feasible, because the ocular surface in our patients had undergone greater insult.

Unfortunately, in cases of herpetic stromal keratitis, usually fewer than 10^4 copies of HSV DNA were detected. This is the same level as that detected in keratitis due to other causes. Therefore, the presence of low levels of HSV DNA alone is not necessarily indicative of herpetic keratitis, and no cutoff value was determined to separate herpetic stromal keratitis from nonherpetic stromal keratitis. To determine whether there really is a threshold level of HSV DNA for diagnosis of HSV as the causative pathogen, more clinical information must be accumulated from various institutions.

In conclusion, real-time PCR is an informative and simple method for diagnosing herpetic keratitis and iridocyclitis and for evaluating possible viral involvement in various ocular inflammatory diseases of the anterior segment. In cases with $>10^4$ copies, the results of real-time PCR alone can be used to reliably diagnose herpetic keratitis. However, in cases with low copy numbers, $<10^4$ copies, diagnosis based on the findings of real-time PCR alone is not recommended. In these cases, real-time PCR must be accompanied by the evaluation of clinical manifestations and drug responsiveness to estimate the extent of HSV participation in the pathogenesis of these cases.

Acknowledgments. We thank Dr. Duko Hamasaki for editing this article. This work was supported by Grant-in-Aid 15390531 for Scientific Research from the Japanese Ministry of Education, Culture, Science, Sports and Technology.

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Jpn J Ophthalmol 2008;52:151–161
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DOI 10.1007/s10384-008-0517-y

CLINICAL INVESTIGATION

Preoperative Disinfection of the Conjunctival Sac with Antibiotics and Iodine Compounds: A Prospective Randomized Multicenter Study

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Abstract

Purpose: To compare disinfection by povidone–iodine (PI) solution with that by polyvinyl alcohol–iodine (PAI) solution in preoperative eyewash and to assess the optimal duration of the preoperative application of 0.5% levofloxacin (LVFX) ophthalmic solution.

Methods: Two-hundred-seventy-two elderly cataract patients who had undergone phacoemulsification aspiration with intraocular lens insertion at 12 clinical facilities in Japan were randomized into three groups: 3-day, 1-day, and 1-h preoperative application of 0.5% LVFX ophthalmic solution. Each LVFX group was further divided into two subgroups; the patients in one subgroup were treated with diluted PI eyewash, and those in the second subgroup with diluted PAI eyewash, immediately before the surgery. Thus, a total of six groups were studied. Conjunctival scrapings were collected four times: before LVFX application, after LVFX application, after the eyewash with iodine compounds, and after the surgery. Samples of the aqueous humor were collected immediately after the surgery. All samples were cultured aerobically and anaerobically.

Results: The 3-day application of LVFX had a significantly higher disinfection rate than the 1-day or 1-h applications ($P < 0.05$). The disinfection rate of PI was 78.0%, and that of PAI was 79.4%; PAI was not inferior to PI as a preoperative disinfectant. However, new isolates, mainly *Propionibacterium acnes* and *Staphylococcus epidermidis*, were detected after LVFX application and even after use of the eyewash with iodine compounds.

Conclusion: Three-day administration of LVFX ophthalmic solution and use of an eyewash with diluted iodine compounds is effective for preoperative disinfection. However, complete elimination of bacteria from the conjunctival sac is difficult. **Jpn J Ophthalmol** 2008;52:151–161 © Japanese Ophthalmological Society 2008

Key Words: levofloxacin, multicenter study, polyvinyl alcohol–iodine, povidone–iodine, preoperative disinfection

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A complete list of the members of the Preoperative Disinfection Study Group follows the Acknowledgments.

Introduction

Recent technical advances in ocular surgery, especially cataract surgery, have provided many patients with excellent vision. However, 100% safety is expected by our patients, and complications threatening visual function must be

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totally eliminated from our surgery. Postoperative endophthalmitis is the most devastating complication and may lead to blindness. Because of the great clinical significance of this complication, effective pre- and postoperative disinfection is essential to prevent the occurrence of infections associated with ocular surgery. However, few disinfection regimens have been based on clear evidence.

In 2002, Ciulla et al.[1] assessed bacterial endophthalmitis prophylaxis techniques commonly used in cataract surgery based on a systematic literature review and evidence rating. None of the prophylactic techniques received the highest of the three possible clinical recommendations (A, crucial to clinical outcome), and only a preoperative povidone–iodine (PI) preparation received the intermediate clinical recommendation (B, moderately important to clinical outcome).[2-5] All other reported prophylactic interventions, including postoperative subconjunctival antibiotic injection, preoperative lash trimming, preoperative saline irrigation, application of preoperative topical antibiotics, antibiotic irrigating solutions, and intraoperative heparin received the lowest clinical recommendation (C, possibly relevant but not definitely related to clinical outcome), because the evidence justifying their use was weak and often conflicting.

In June 2001, a sudden notice from the Japanese pharmaceutical company Meiji Seika Kaisha, whose PI product is used in Japan, confounded ophthalmologists in Japan. This notice stated that PI is more toxic and allergenic in ophthalmic use than when used on skin or other mucosa, and it should not be used for ocular surface irrigation. Since then, we ophthalmologists in Japan must use preoperative PI on our own responsibility and ignore the manufacturer's warning. Therefore, the only evidence-based method that prevents the most devastating complication of ocular surgery cannot be used legally.

However, another generic iodine compound, polyvinyl alcohol–iodine (PAI), has been developed that is permitted for ophthalmic use in Japan. In this compound, iodine is combined with polyvinyl alcohol instead of with polyvinyl pyrrolidone as in PI. Theoretically, PAI should have a disinfective capability similar to that of PI. However, its efficacy in preoperative disinfection has not been clinically tested.[6]

Although preoperative topical antibiotics were ranked as the least recommended clinically in the study by Ciulla et al.,[1] several reports have indicated that preoperative bacterial flora can be reduced by topical antibiotics.[7-11] In Japan, topical antibiotics are widely used preoperatively; however, the appropriate duration of their application is unclear.

Considering this background, the Japanese Association for Ocular Infection (JAOI) decided to make an official JAOI recommendation regarding preoperative disinfection methods. To obtain evidence for this recommendation, JAOI designed a multicenter study of preoperative disinfection regimens. This multicenter study had two objectives. The first was to compare disinfection between a PI solution and a PAI solution used as a preoperative eyewash, and the second was to determine the optimal duration of the preoperative application of an antibiotic ophthalmic solution containing levofloxacin (LVFX), which was chosen as a representative drug.

Patients and Methods

Patient Selection

A total of 365 patients with age-related cataract 60 years old or older who were scheduled for cataract surgery by phacoemulsification aspiration (PEA) with intraocular lens (IOL) implantation participated in this study. This prospective randomized multicenter study was performed at 12 clinical facilities in Japan (Table 1). In cases of bilateral cataract surgery, the first eye was used in the analyses. After approval by the Institutional Review Board at each clinical facility, voluntary written consent for their participation was obtained from all of the patients. Patients with the following conditions were excluded: (1) those with a discharge of pus accompanying lacrimal sac irrigation; (2) those undergoing combined surgery such as glaucoma operation/keratoplasty; (3) those with intraoperative complications; (4) those with poor pupillary dilatation; (5) those in whom the usual PEA procedures would be difficult to perform; (6) those judged by the investigator to be otherwise inappropriate as subjects; (7) those with a history of hypersensitivity to iodine compounds or fluoroquinolone drugs; and (8) those who had received antibacterial drugs systemically or topically within 2 weeks before the start of the study.

Allocation of Patients

The patients were randomly allocated to the following six groups using a central registration method via the Internet. Three groups received 0.5% LVFX ophthalmic solution preoperatively at different times as follows: a single application 1 h before surgery (1-h application group); three applications on the day before surgery plus a single application 1 h before surgery (1-day application group); and applications three times a day for 3 days before the day of surgery plus a single application 1 h before surgery (3-day application group). Each group was further subdivided into two

Table 1. Participating facilities

Hokkaido University Graduate School of Medicine
University of Tsukuba
Tokyo Medical University Kasumigaura Hospital
Tokyo Medical University
Tokyo Medical University Hachioji Medical Center
Hatano Eye Clinic
Kyoto Prefectural University of Medicine
Kinki University School of Medicine
Tottori University
The University of Tokushima Graduate School
Tokushima Red Cross Hospital
Ehime University School of Medicine

subgroups according to the type of preoperative eyewash solution used, a PI group and a PAI group, for a total of six groups.

Methods of Disinfection

LVFX ophthalmic solution was instilled at the designated times in each group. The PI and PAI solutions were diluted immediately before use with physiological saline to make a 16-fold diluted PI solution and a sixfold diluted PAI solution for the eyewash disinfection. One minute after skin disinfection with undiluted PI solution, eyewash disinfection was performed with 50 ml of the diluted iodine compound, using a syringe without a needle. The eyelids were not everted, and the lacrimal sac was not compressed. The solution was not rinsed off after the eyewash application, and a sterile gauze was immediately pressed down over the eye to absorb the residual eyewash solution. Then the sterile gauze was draped carefully around the eyelids.

Methods of Sampling

Conjunctival sac scrapings were collected at the following four designated times as specimens for bacteriological examination: (1) prior to initial administration of LVFX ophthalmic solution (samples were collected from 7 days before to immediately before the initial administration); (2) after the final administration of LVFX ophthalmic solution and before skin disinfection immediately prior to surgery; (3) after eyewash administration and between the gauze application and draping; and (4) immediately following the surgery after the removal of the eyelid speculum. In addition 0.05 ml of aqueous humor was aspirated for bacteriological examination from the side port following the surgery and before the final collection of conjunctival sac scrapings.

The conjunctival sac scrapings were collected after anesthetizing the conjunctiva with preservative-free oxybuprocaine hydrochloride 0.4% ophthalmic solution by inserting a swab moistened with sterile physiological saline into the fornix of the lower eyelid and passing it once back and forth. The swab was placed into a media named ANA Port Microbiol Lab [Research Foundation for Microbial Diseases of Osaka University; composition of the media: solution I (75 ml), solution II (75 ml), 0.1% resazurin solution (1.0 ml), 8% sodium carbonate solution (37.5 ml), 5% cysteine hydrochloride solution (10 ml), glycerol (100 ml), agar (1 g), Lab-lemco powder (10 g), and distilled water (900 ml); solution I: KH_2PO_4 (0.3%), NaCl (0.6%), $(NH_4)_2SO_4$ (0.3%), $CaCl_2$ (0.03%), and $MgSO_4$ (0.03%); solution II: KH_2PO_4 (0.3%)]. For the conjunctival scrapings collected after use of the eyewash, 0.01% sodium thiosulfate was added to the ANA Port Microbiol Lab to neutralize the iodine. The specimens were frozen within 1 h of collection and stored. The 0.05-ml aqueous humor samples were collected from the side port following surgery with a 1-ml syringe with a 27-gauge blunt needle attached, injected into the ANA Port Microbiol Lab, and frozen and stored within 1 h.

Bacteriological Examination

The stored frozen specimens were sent to the Research Foundation for Microbial Diseases of Osaka University. This facility performed aerobic and anaerobic cultures and carried out identification tests and drug susceptibility tests. Although the primary objective of this study was to carry out bacteriological examinations, cultures were also prepared for culturing and identification of fungi. Clinical thioglycolate media plates were incubated at 36.5°C for 5 days to stimulate aerobic and microaerophilic bacteria growth. The Columbia agar plates containing 0.5% sheep's blood were placed in an anaerobic bag for isolation of anaerobic bacteria at 36.5°C for 5 days and incubated at 36.5°C for 2 days with 5% carbon dioxide. Sabouraud's agar plates were incubated at 30°C for 1 week under aerobic conditions for the growth of fungi.

Allowed and Prohibited Materials for the Operation

The use of a silicon, acryl, or one- or three-piece poly(methyl methacrylate) (PMMA) IOL was allowed, and it was left to the doctor in charge to decide whether to use an injector for inserting the IOL. In addition, all kinds of intraocular irrigating solution and viscoelastic materials were allowed. Concomitant use of anesthetics, infusions, mydriatic or miotic agents, and analgesics were allowed, but topical or systemic administration of antibiotics and antimicrobials were prohibited until the completion of the operation.

Postoperative Monitoring of Patients

The general postoperative course was monitored at 2 weeks and 1 month to determine the effect of prophylactic measures on postoperative infections. Some patients were monitored until 6 months postoperatively. The course was classified as (1) Particularly favorable (early disappearance of hyperemia, aqueous humor inflammation); (2) Favorable; (3) Slightly unfavorable (delayed disappearance of hyperemia, aqueous humor inflammation); or (4) Suspected postoperative infection.

Primary End Points and Statistical Analyses

One of the primary end points was to find the two-sided confidence interval (CI) of the culture-negative conversion rates in the PI and PAI groups. Equivalence was verified by using a confidence coefficient of 95% and an equivalence margin of 10%. As one more primary end point, the culture-negative conversion rate of each group of preoperative application LVFX was obtained for comparison among the

three groups. A Cochran-Mantel-Haenszel test was performed by using the bacterial retention ratio as the response variable and the PI group/PAI group as the stratum. Also, contrast tests were performed with a logistic model to detect significant differences between the groups. Furthermore, a χ-squared test was performed to compare patient characteristics and surgical findings, the incidence of new isolates after LVFX application and used of the PI or PAI eyewash, and the postoperative course at 2 weeks and 1 month after surgery for each of the preoperative disinfection methods. The analysis software used was SAS (SAS Institute, Cary, NC, USA).

Results

Patients Enrolled

Of the 365 registered and allocated patients, bacteriological test specimens were taken from 314 (86.0%). There were 51 patient discontinuations (14.0%). The reasons for the discontinuations included patient request (26 patients, accounting for the majority of discontinuations), suspension of the operation (five), posterior capsular rupture (five), a change in the surgical procedures (four), poor general condition (three), and a zonular break (two). Of the 314 patients sampled, 272 were assessed for disinfection efficacy, of which 244 (89.7%) were bacteria-positive prior to the disinfection procedure and 28 (10.3%) were bacteria-negative. Forty-two patients were excluded from the efficacy assessment: 18 patients were mistakenly given antibiotics before and during the operation; in 17 patients sampling errors were made during collection of the specimens; and in seven patients errors in the administration of the LVFX ophthalmic solution were made.

Patient Characteristics

Of the 244 patients with positive culture prior to any application of LVFX ophthalmic solution, 76 were in the 1-h application group, 89 in the 1-day application group, and 79 in the 3-day application group. Of these 244 patients, 118 patients were in the PI group and 126 in the PAI group. The 244 patients with a positive culture prior to LVFX application had a median age of 74 (60–95) years, and 46.3% were men and 53.7% were women. The patient characteristics and surgical findings are summarized in Tables 2 and 3. Apart from the significant difference ($P = 0.01$) in incision type among the three LVFX ophthalmic solution application groups, no difference was found among these three groups or between the two eyewash solution groups with respect to any of the factors.

Microbes Detected before Application of LVFX

The microbe strains in the 244 patients showing a positive culture prior to LVFX application were *Staphylococcus epidermidis* (31.5%), *Propionibacterium acnes* (27.5%), *Corynebacterium* spp. (9.7%), *Staphylococcus aureus* (5.2%), *Enterococcus faecalis* (2.4%), *Streptococcus oralis* (1.7%), *Propionibacterium avidum* (1.7%), *Propionibacterium granulosum* (1.4%), *Staphylococcus mitis* (1.2%), *Staphylococcus lugdunensis* (1.2%), and others (16.6%) (Table 4).

Serial Changes in the Microbial Isolation Rate by LVFX Application Group in Patients with Positive Cultures before LVFX Application

The changes in the percentage of positive cultures following the initial administration of LVFX ophthalmic solution are shown in Fig. 1. In this analysis, only the conversion of isolates at the first sampling before LVFX isolation was examined; therefore, newly isolated bacteria after LVFX isolation were not included.

The rates of positive culture in the conjunctival scrapings after the final LVFX application were 59.2% in the 1-h application group, 49.4% in the 1-day application group, and 39.2% in the 3-day application group. The three groups differed significantly ($P = 0.0131$; Cochran-Mantel-Haenszel test). In contrast, logistic model tests showed a difference between the 3-day application group and 1-h application

Table 2. Patient characteristics

Patient characteristics	Eyewash solution			Preoperative application of LVFX ophthalmic solution			
	PI group ($n = 118$)	PAI group ($n = 126$)	P	1-h application group ($n = 76$)	1-day application group ($n = 89$)	3-day application group ($n = 79$)	P
Age (years)	73.3 ± 5.88	73.6 ± 7.37	0.769	74.0 ± 7.33	72.5 ± 6.62	74.0 ± 6.03	0.244
Sex ratio							
Male	43.22	49.21	0.420	56.58	44.94	37.97	0.066
Combined disease							
Glaucoma	6.78	6.35	1.000	6.58	7.87	5.06	0.766
Diabetes	16.95	12.7	0.450	15.79	13.48	15.19	0.923
Dry eye	1.69	2.38	1.000	1.32	0	5.06	0.060
Allergic disease	0	0.79	1.000	0	0	1.27	0.635

Age is given as mean ± SD. Categorical data are given as percentages. All P values were calculated with the χ-squared test.
LVFX, levofloxacin; PAI, polyvinyl alcohol–iodine; PI, povidone–iodine.

Table 3. Operative findings

Operative findings	Eyewash solution			Preoperative application of LVFX ophthalmic solution			
	PI group ($n = 110$)	PAI group ($n = 121$)	P	1-h application group ($n = 72$)	1-day application group ($n = 85$)	3-day application group ($n = 74$)	P
Incision site							
Upper	80.0	71.1	0.156	70.8	74.1	81.1	0.334
Temporal	20.0	28.9		29.2	25.9	18.9	
Incision type							
One side sclerocorneal	8.2	5.8	0.579	12.5	8.2	0.0	0.010
3 sides sclerocorneal	89.1	89.3		81.9	88.2	97.3	
Clear cornea	2.7	5.0		5.6	3.5	2.7	
Incision size							
<3.0 mm	5.5	1.7	0.101	4.2	3.5	2.7	0.937
3.0 mm	25.5	28.9		23.6	29.4	28.4	
3.5 mm	24.6	14.1		20.8	20.0	16.2	
4.0 mm	40.9	48.8		48.6	41.2	46.0	
>4.0 mm	3.6	6.6		2.8	5.9	6.8	
Handling of nucleus							
Phaco chop	51.8	47.1	0.452	51.4	50.6	46.0	0.591
Divide & Conquer	45.5	52.1		47.2	49.4	50.0	
Pre chop	1.8	0.0		0.0	0.0	2.7	
Others	0.9	0.8		1.4	0.0	1.4	
Sclerocorneal suture							
Yes	30.9	39.7	0.211	34.7	37.7	33.8	0.870
IOL							
Silicon	1.8	1.7	0.217	2.8	1.2	1.4	0.677
Acryl	80.0	68.6		72.2	76.5	73.0	
1P-PMMA	3.6	5.0		1.4	4.7	6.8	
3P-PMMA	14.6	24.8		23.6	17.7	18.9	
Use of Injector							
Yes	37.3	34.7	0.789	33.3	36.5	37.8	0.843
Irrigating solution							
BSS Plus	76.4	79.3	0.382	76.4	76.5	81.1	0.296
Opeguard MA	0.0	1.7		0.0	0.0	2.7	
Opeguard Neo Kit	23.6	19.0		23.6	23.5	16.2	
Viscoelastic substance							
HEALON	27.3	23.1	0.416	23.6	25.9	25.7	0.961
HEALON V	0.9	2.5		2.8	2.4	0.0	
OPEGAN	15.5	14.0		16.7	15.3	12.2	
OPEGAN Hi	52.7	44.6		44.4	49.4	51.4	
OPELEAD	0.9	0.0		1.4	0.0	0.0	
VISCOAT	2.7	5.0		4.2	3.5	4.1	
HYALUOPE	14.5	23.1		22.2	16.5	18.9	
Operation time (min)	23.9 ± 13.5	22.3 ± 12.9	0.343	22.1 ± 14.5	24.4 ± 12.8	22.5 ± 12.2	0.501
Volume of Irrigating solution (ml)	213.3 ± 100.5 (65)	215.7 ± 121.9 (73)	0.889	203.2 ± 113.6 (39)	225.9 ± 115.1 (57)	209.3 ± 107.2 (42)	0.587

Operation time and volume of irrigating solution are given as mean ± SD. Categorical data are given as percentages. All P values were calculated with the χ-squared test.
IOL, intraocular lens; 1P, one piece; 3P, three piece; PMMA, poly(methyl methacrylate).

group, but no significant difference between the 3-day and 1-day application groups.

After use of the eyewash with iodine compounds, the rates of positive culture were 27.6% in the 1-h application group, 25.8% in the 1-day application group, and 10.1% in the 3-day application group. The three groups differed significantly ($P = 0.0076$; Cochran-Mantel-Haenszel test). In addition, the logistic model test showed significant differences between the 3-day and 1-day application groups, as well as between the 3-day and 1-h application groups. After the eyewash, a synergistic effect was achieved from the application of LVFX and iodine compounds, and this effect was more prominent in the 3-day application group than in the 1-h or 1-day groups.

Bacterial cultures in the postoperative aqueous humor were positive in only four patients (5.1%) in the 3-day application group. No characteristic features were noted in the patient profiles or surgical findings of these four patients.

Table 4. Microbes detected before the application of LVFX

Species	No. of strain	%
Staphylococcus epidermidis	133	31.5
Propionibacterium acnes	116	27.5
Corynebacterium spp.	41	9.7
Staphylococcus aureus	22	5.2
Enterococcus faecalis	10	2.4
Streptococcus oralis	7	1.7
Propionibacterium avidum	7	1.7
Propionibacterium granulosum	6	1.4
Streptococcus mitis	5	1.2
Staphylococcus lugdunensis	5	1.2
Others	70	16.6
Total	422	100.0

The percentages of positive culture in the postoperative conjunctival scrapings were 17.1% in the 1-h application group, 20.0% in the 1-day application group, and 13.9% in the 3-day application group. The three-day application group had the lowest positive rate; however, there was no significant difference among the three groups.

Serial Changes in the Microbial Isolation Rate by the Type of Iodine Compound in the Eyewash

The changes in the rates of positive culture by eyewash solution are shown in Fig. 2. The rates of positive culture in the conjunctival scrapings after eyewash decreased to 22.0% in the PI group and to 20.6% in the PAI group. More specifically, the disinfection rates in the conjunctival scrapings after the eyewash were 78.0% (95% CI; 69.4%–85.1%) in the PI group and 79.4% (95% CI; 71.3%–86.1%) in the PAI group; the difference in the disinfectant rate was –1.4% (–11.7%–8.9%). Therefore, an equivalence in the disinfection effect of the PI and PAI groups could not be verified, but the CIs showed a high degree of overlap, and it was verified that PAI was not inferior to PI. Furthermore, the rate of positive culture from the postoperative aqueous humor was 1.7% (2/118 eyes) in the PI group and 1.6% (2/126 eyes) in the PAI group, and that from the postoperative conjunctival scrapings was 17.8% (21/118 eyes) in the PI group and 16.7% (21/126 eyes) in the PAI group, showing no difference between the two iodine eyewash compounds.

New Isolates After Preoperative LVFX Application

New isolates were detected after preoperative application of LVFX ophthalmic solution that were not detected before

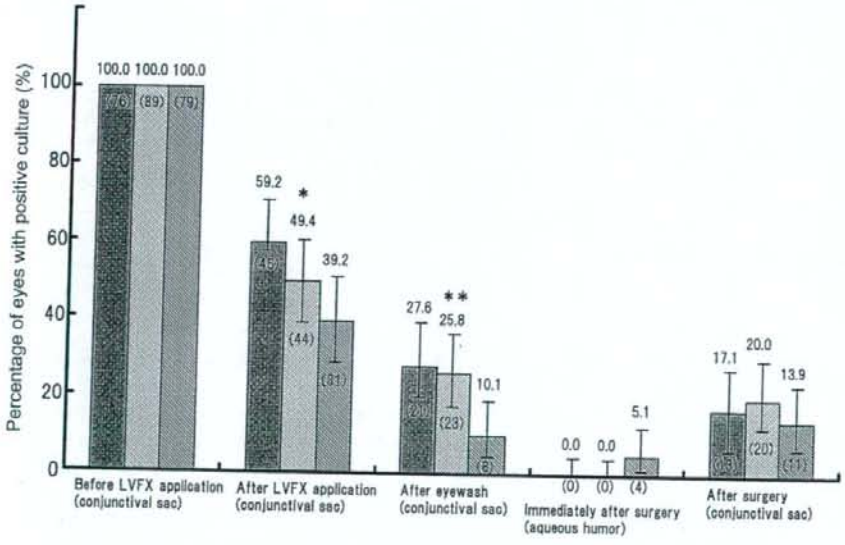

Figure 1. Serial changes in the microbial isolation rate in relation to the levofloxacin (*LVFX*) application period in the patients with positive cultures before LVFX application: ▩, 1-h application of LVFX; ▨, 1-day application of LVFX; ▧, 3-day application of LVFX. Eyes with any single isolate were counted each time at the scheduled assessment time points. Eyes with new microbe isolates were not counted. The number in parentheses on each bar is the number of eyes with positive culture. $^*P < 0.05$; $^{**}P < 0.01$ (Cochran-Mantel-Haenszel test).

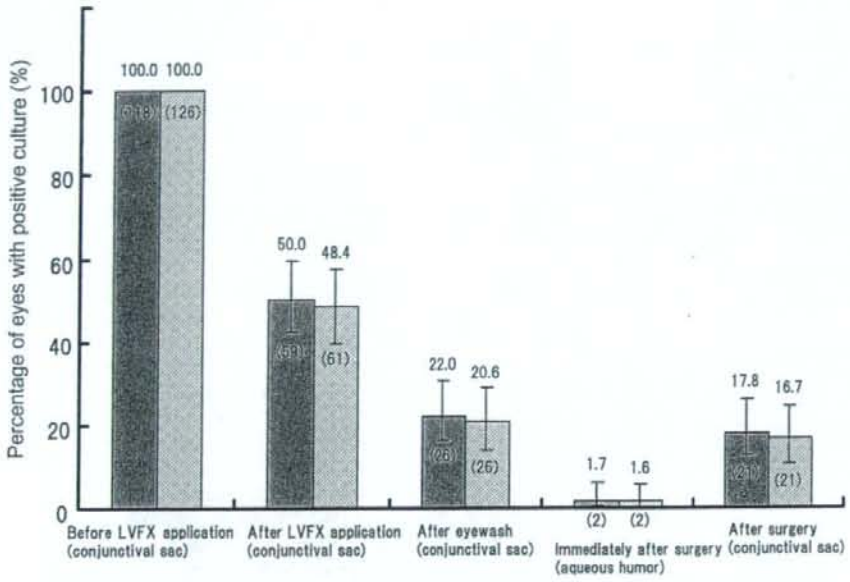

Figure 2. Serial changes in the microbial isolation rate in relation to the type of iodine compound used for eyewash: ■, PI group; □, PAI group. Eyes with any single isolate were counted each time at the scheduled assessment time points. Eyes with new microbe isolates were not counted. The number in parentheses on each bar is the number of eyes with positive culture.

Table 5. New isolates detected after preoperative LVFX application

New isolates[a]	Preoperative application of LVFX ophthalmic solution			
	1-h group	1-day group	3-day group	Total
Propionibacterium acnes	12	16	14	42
Staphylococcus epidermidis	10	5	3	18
MSSA	5	0	3	8
Corynebacterium spp.	3	3	0	6
Staphylococcus hominis	4	0	0	4
Micrococcus luteus	3	0	1	4
Peptostreptococcus spp.	2	1	0	3
Streptococcus mitis	0	0	2	2
Streptococcus oralis	2	0	0	2
α-*Streptococcus*	0	0	2	2
MRSA	2	0	0	2
Staphylococcus capitis	0	2	0	2
Staphylococcus lugdunensis	1	1	0	2
Anaerobic Gram-negative bacillus	2	0	0	2
Others	5	4	2	11
Total	51	32	27	110

[a]New isolates were detected after preoperative application of the LVFX ophthalmic solution and were not detected before LVFX application.
MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *S. aureus*.

LVFX application at baseline observation (Table 5). The main new isolates were *Propionibacterium acnes* and *Staphylococcus epidermidis*, which were the same as those isolated in other eyes before LVFX application. The incidence rate of new isolates in the 1-h application group (43.8%; 39/89) was significantly higher than that in the 1-day (28.4%; 27/95) or 3-day (28.4%; 25/88) application groups (P = 0.0445, χ-squared test).

New Isolates After Eyewash with Iodine Compounds

New isolates were detected after eyewash with PI or PAI that were not detected before LVFX application at baseline observation (Table 6). The number of new isolates detected after eyewash was decreased compared with after preoperative LVFX application. The main new isolates were the same, *Propionibacterium acnes* and *Staphylococcus epider-*

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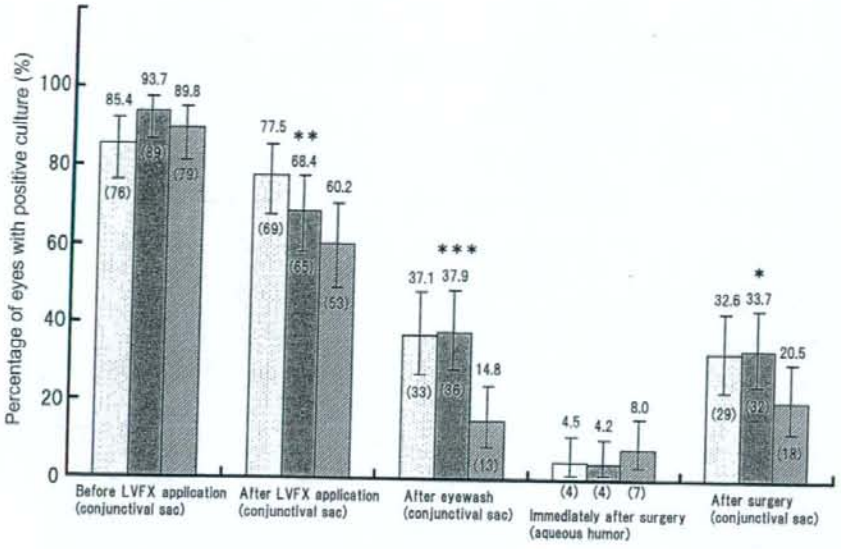

Figure 3. Serial changes in microbial isolation rate in relation to the LVFX application period and including patients with positive or negative cultures before LVFX application: □, 1-h application of LVFX; ■, 1-day application of LVFX; ▨, 3-day application of LVFX. Eyes with any single isolate are counted each time at the scheduled time points of assessment. Eyes with new microbe isolates are also counted. The number in parentheses on each bar is that the number of eyes with positive culture. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Cochran-Mantel-Haenszel test).

Table 6. New isolates detected after eyewash with iodine compounds

New isolates[a]	PI group	PAI group	Total
Propionibacterium acnes	6	7	13
Staphylococcus epidermidis	6	4	10
Streptococcus oralis	0	3	3
Streptococcus mitis	1	1	2
Staphylococcus aureus	0	2	2
Micrococcus luteus	1	1	2
Aerobic Gram-positive bacillus	0	2	2
Enterococcus casseliflavas	1	0	1
Staphylococcus xylosus	0	1	1
Micrococcus lylae	0	1	1
Corynebacterium sp.	1	0	1
Bacillus sp.	1	0	1
Klebsiella pneumoniae	1	0	1
Moraxella osloensis	0	1	1
Actinomyces sp.	1	0	1
Rhodotorula glutinis	1	0	1
Total	20	23	43

[a]New isolates were detected after the eyewash with iodine compounds and were not detected before the eyewash.

midis. The incidence rates of new isolates in the PI and PAI groups were 13.4% (18/134) and 14.5% (20/138), and the two groups did not differ significantly.

Serial Changes in the Microbial Isolation Rate Stratified by the Duration of LVFX Application, Including Patients with Positive and Negative Cultures before LVFX Application

The changes in the percentage of positive cultures by the duration of the administration of LVFX ophthalmic solution in 272 eyes are shown in Fig. 3. All patients with positive or negative cultures before LVFX application are included, and all eyes with new isolates as well as bacteria detected at any earlier sampling points were evaluated as eyes with positive culture. The rates of positive cultures from the conjunctival scrapings after the final preoperative application of LVFX ophthalmic solution were 77.5% in the 1-h application group, 68.4% in the 1-day application group, and 60.2% in the 3-day application group. The three groups differed significantly ($P = 0.0077$; Cochran-Mantel-Haenszel test). In addition, logistic model tests showed a significant difference between the 3-day and 1-h application groups, but no significant difference between the 3-day and 1-day application groups.

After use of the eyewash with the iodine compounds, the rates of positive culture were 37.1% in the 1-h application group, 37.9% in the 1-day application group, and 14.8% in the 3-day application group. The three groups differed significantly ($P = 0.0007$; Cochran-Mantel-Haenszel test). Logistic model tests showed significant differences both between the 3-day and 1-day application groups, and between the 3-day and 1-h application groups.

The rates of positive cultures from the postoperative conjunctival scrapings were 32.6% in the 1-h application group, 33.7% in the 1-day application group, and 20.5% in the 3-day application group, showing a significant difference among the three groups ($P = 0.0490$; Cochran-Mantel-Haenszel test).

Postoperative Monitoring of Patients

No patient suspected of having postoperative infection was found during postoperative monitoring, either at 2 weeks or 1 month after surgery. The postoperative findings for the LVFX application groups showed that most patients were

Table 7. Observation of postoperative infections

	Eyewash solution					Preoperative application of LVFX ophthalmic solution						
	PI group		PAI group			1-h application group		1-day application group		3-day application group		
Postoperative infection	%	*n*	%	*n*	*P*	%	*n*	%	*n*	%	*n*	*P*
2 weeks after surgery ($n = 255$)												
Particularly favorable	24.4	30	25.8	34	0.963	25.3	21	24.2	22	25.9	21	0.661
Favorable	74.0	91	72.0	95		72.3	60	72.5	66	74.1	60	
Slightly unfavorable	1.6	2	2.3	3		2.4	2	3.3	3	0.0	0	
Suspected postoperative infection	0.0	0	0.0	0		0.0	0	0.0	0	0.0	0	
One month after surgery ($n = 253$)												
Particularly favorable	22.0	27	22.3	29	0.303	22.0	18	24.4	22	19.8	16	0.185
Favorable	78.1	96	75.4	98		78.0	64	72.2	65	80.2	65	
Slightly unfavorable	0.0	0	2.3	3		0.0	0	3.3	3	0.0	0	
Suspected postoperative infection	0.0	0	0.0	0		0.0	0	0.0	0	0.0	0	
6 months after surgery ($n = 139$)												
Particularly favorable	10.4	7	8.3	6	0.511	6.4	3	10.6	5	11.1	5	0.337
Favorable	89.6	60	88.9	64		93.6	44	85.1	40	88.9	40	
Slightly unfavorable	0.0	0	2.8	2		0.0	0	4.3	2	0.0	0	
Suspected postoperative infection	0.0	0	0.0	0		0.0	0	0.0	0	0.0	0	

All *P* values were calculated with the χ-squared test.

assessed as "Particularly favorable" or "Favorable," with a very small percentage being assessed as "Slightly unfavorable" at all monitoring times. No significant difference among the three LVFX application groups was found at any monitoring time (2 weeks postoperatively, $P = 0.661$; 1 month postoperatively, $P = 0.185$; 6 months postoperatively, $P = 0.337$; χ-squared test) (Table 7). The postoperative findings according to the eyewash solution used showed that there was no significant difference between the two groups at any monitoring time (2 weeks postoperatively, $P = 0.963$; 1 month postoperatively, $P = 0.303$; 6 months postoperatively, $P = 0.511$; χ-squared test).

Discussion

Our results showed that the disinfection effect of PAI was not significantly different from that of PI preoperatively, although the effective iodine concentration of the 16-fold-diluted PI were almost double that of 6-fold-diluted PAI (0.063% vs. 0.033%). Berkelman et al.[12] reported that diluted PI was more effective than full-strength PI in experiments in which the bacterial suspension was mixed directly with the PI solution. In their study, the maximum effect was achieved with a between tenfold and 100-fold dilution of PI, namely, between 0.1% and 0.01% of effective iodine concentration. The concentrations of both diluted compounds in our study were within this level of effective concentration. In the United States, a twofold dilution of PI has been used, which is theoretically too high to achieve appropriate disinfection.

Although the percentage of ocular surface disinfection in the PAI group was higher than that in the PI group, there was no significant difference between the two groups (data not shown). No serious epithelial problems were found in the eyes receiving the PAI preparations.

Our results also showed that a 3-day application of preoperative LVFX ophthalmic solution was significantly more effective than 1-h or 1-day applications. Grimes et al.[9] reported that a 3-day course (one drop four times a day for 3 days) of topical gentamicin 0.3% attained lower colony counts in the conjunctiva compared with a half-day course (one drop every 3 h the night before and the morning of surgery). Ta et al.[10] reported that the application of topical ofloxacin for 3 days before surgery is more effective in eliminating bacteria from the conjunctiva than an application 1 h before surgery. Kaspar et al.[11] also found in their prospective randomized study that a 3-day application of topical ofloxacin reduces the contamination rate of microsurgical knives in cataract surgery more effectively than a 1-h application. These previous data together with our results strongly support the idea that the 3-day application of antibiotics is a better way to reduce bacterial amounts in the conjunctiva preoperatively than a shorter application course. Also, the synergistic effects of preoperative topical antibiotics and iodine compounds, which were indicated in a previous report,[8] were more prominent in the 3-day application of LVFX than in 1-day or 1-h applications, according to the bacterial isolation rate data after eyewash in our study. This result is reasonable, because the residual bacteria before use of the eyewash with iodine compounds were fewer in the 3-day application of LVFX than in the 1-day or 1-h applications, and the additional effect caused by iodine compound irrigation became more apparent in the 3-day application of LVFX.

The possibility that the prolonged use of prophylactic antibiotics might encourage the appearance of microorganisms not normally present or resistant to antibiotics is of