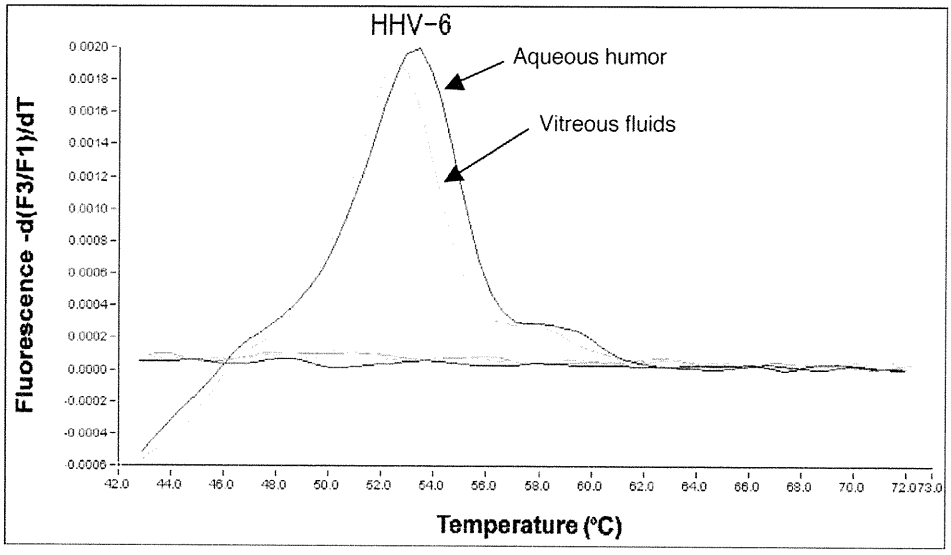

FIGURE 3. Results for multiplex PCR in a patient with HHV-6–positive uveitis. A significant positive curve was seen at 52°C, indicating detection of HHV-6 genomic DNA in the ocular fluids (case 1). DNA from other herpes viruses, such as HSV1, HSV2, VZV, EBV, CMV, HHV7, and HHV8, was not detected in this sample.

DNA, and two cases were found to be HHV-6 type A and six cases were type B. In addition, HHV-6 mRNA was detected in intraocular samples from HHV-6–positive patients, suggesting that viral replication or reactivation may occur in the eye.

Recently, Cohen et al.[5] reported that HHV-6A DNA could be detected by PCR in vitreous fluid from a patient with CMV-associated retinitis when vitreous fluids were assayed from 101 patients with ocular inflammation for HHV-6A, HHV-6B, and HHV-7. HHV-6B DNA was also detected in vitreous fluid from a patient with idiopathic uveitis in the absence of CMV DNA. This study suggests that HHV-6A and HHV-6B DNA are detectable in approximately 1% of vitreous samples from patients with ocular inflammation. In our study, we show that HHV-6 DNA was detectable in 2% of ocular samples from patients with intraocular inflammation following screening for HHV-1 to –8 infection using multiplex PCR.

In a previous study,[16] we found that intraocular HHV DNA was detectable in a wide range of herpes virus–associated uveitis cases when analysis was performed using multiplex PCR. PCR is a valuable tool for the diagnosis of herpetic uveitis and it is now possible to exclude nonherpetic uveitis patients using this method. Moreover, de Boer et al.[8] previously found that in patients with herpetic anterior uveitis, PCR was more frequently positive than the Goldmann-Witmer coefficient. HHV-6 has been implicated in ocular inflammation, most remarkably when the posterior segment of the eye was affected.[6,7,10–12] On the other hand, the role of HHV-6 as a cause of anterior uveitis is inconclusive and further studies are required. As revealed in this study, we found three cases of anterior inflammatory diseases including keratitis and five cases of pan- or posterior inflammatory diseases in the eye.

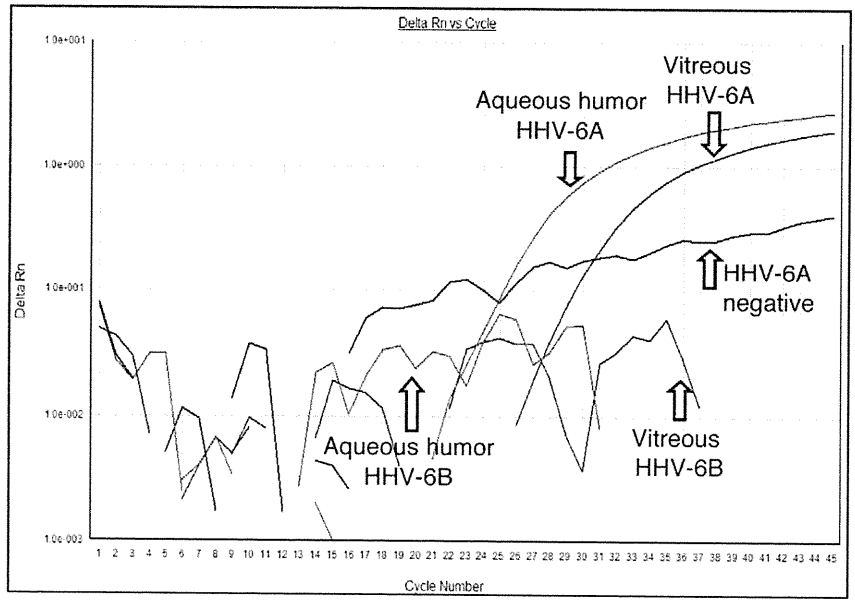

FIGURE 4. Detection of HHV-6 DNA by quantitative real-time PCR. The real-time PCR results for the samples from case 1 showed that intraocular samples, such as aqueous humor and vitreous fluids, contained a high copy number of HHV-6A DNA, but not HHV-6B DNA.

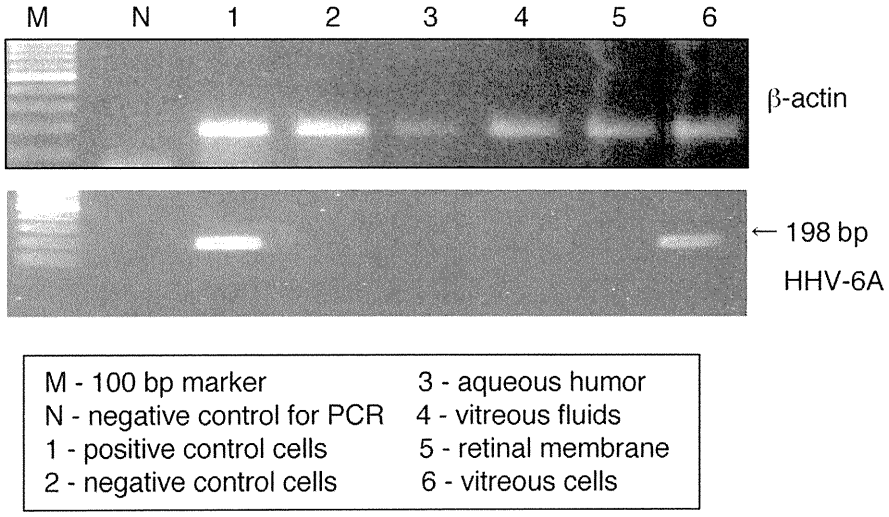

FIGURE 5. Detection of HHV-6 mRNA in intraocular samples. HHV-6A mRNA was detected in samples from vitreous cells, but other ocular samples, such as aqueous humor, vitreous fluids, and retinal membrane tissues were all negative (*lower image*). All samples, including control RNA, were positive for β-actin (*upper image*).

The detection of HHV-6 in the eye might not be clinically relevant. HHV-6 can latently reside in cells of the lymphoid and myeloid lineage and it may have entered the inflamed eye via immune cells, similar to EBV and human immunodeficiency virus.[3,22,23] Thus, HHV-6 DNA has been detected in circulating T cells, monocytes, and leukocytes and may simply have been carried into the eyes in the inflammatory cells as a result of destruction of the blood-retina barrier. Our data indicate that most HHV-6 DNA in intraocular fluids of inflamed eyes might be a consequence of the release of HHV-6 DNA from resident ocular cells caused by intraocular inflammation. A high copy number of HHV-6 DNA was detected in patients with severe ocular inflammation, pan- or posterior uveitis, or endophthalmitis (Tables 1 and 2). This is supported by the findings of Arao et al.,[24] who showed that HHV-6 can infect human retinal pigment epithelial cells.

We detected HHV-6 in only one patient with an ocular surface inflammatory disorder. The patient was a young healthy donor suffering from atopic dermatitis. Okuno et al.[9] recently reported that 14 of 22 patients with corneal inflammation were positive for HHV-6, suggesting that the association of HHV-6 with disease was more frequent than with other herpes viruses, such as HSV-1. Thus, HHV-6 may be another sole causative agent of corneal inflammation.

HHV-6 reactivation frequently accompanies CMV reactivation,[25] and the presence of HHV-6A DNA in the eye may simply reflect the immunocompromised state of the patient. Case 6 in this study was a patient with CMV retinitis who was also found to be HHV-6A DNA-positive; however, with the exception of this patient, our HHV-6 PCR-positive patients were neither young nor immunosuppressed. We previously used multiplex PCR to search for HHV-6 in ocular fluids from 100 patients with uveitis and detected HHV-6A DNA in one patient with severe unilateral uveitis (case 1).[7] This patient's ocular fluid also contained antibodies to *Toxocara canis* larvae and we finally diagnosed ocular toxocariasis and HHV6-related panuveitis.[7] In this study, 7 patients were found to have other infectious agents, including bacteria, other herpes viruses (HSV-1), and parasites (*Toxocara*); however, it is unclear whether HHV-6 was the predominant pathogen. It is assumed that HHV-6 infections play a secondary role in the pathogenesis of ocular inflammation. Therefore, we tested intraocular samples for the presence of HHV-6 mRNA. Additional tests for HHV-6 RNA or protein in ocular tissues would have been more definitive and provided evidence of HHV-6 replication. We found HHV-6A mRNA and a high copy number of HHV-6 DNA in the same sample from a patient with ocular toxocariasis (case 1). As far as we know, this is the first report of detection of both HHV-6 DNA and mRNA in an ocular sample. The RT-PCR assay can reliably differentiate between latent and actively replicating HHV-6 and its use should allow an insight into the pathogenesis of this ubiquitous virus as previously reported.[21]

In conclusion, ocular samples collected from patients with infectious ocular disorders can contain a high copy number of HHV-6 DNA. The HHV-6-positive case was found to have HHV-6 DNA and mRNA in the inflamed eye. We are currently conducting experiments to determine whether HHV-6 type A and type B can infect ocular cells, such as retinal pigment epithelium, in vitro. Infected ocular cells can produce inflammatory cytokines and chemokines that differ from those in normal uninfected cells.

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INFLAMMATORY DISORDERS

Novel diagnosis of fungal endophthalmitis by broad-range real-time PCR detection of fungal 28S ribosomal DNA

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Abstract
Aim To detect the fungal genome in the ocular fluids of patients with fungal endophthalmitis by using a novel broad-range polymerase chain reaction (PCR) system.
Methods After informed consent was obtained, ocular fluid samples (aqueous humor or vitreous fluids) were collected from 497 patients (76 patients with infectious endophthalmitis including clinically suspected bacterial and fungal endophthalmitis and 421 patients with infectious or non-infectious uveitis). Forty ocular samples from non-infectious patients without ocular inflammation were collected as controls. Fungal ribosomal DNA (28 S rDNA) was measured by a quantitative real-time PCR assay.
Results Fungal 28 S rDNA of the major fungal species, such as *Candida*, *Aspergillus*, and *Cryptococcus*, were detected by novel broad-range real-time PCR examination ($>10^1$ copies/ml). Fungal 28 S rDNA was detected in the ocular fluids of 11 patients with endophthalmitis or uveitis (11/497, 2.2%). All 11 positive samples were detected in the infectious endophthalmitis patients (11/76, 14.5%). These PCR-positive ocular fluids had high copy numbers of fungal 28 S rDNA (range, 1.7×10^3 to 7.9×10^6 copies/ml), which indicated the presence of fungal infection. Of the 11 patients who were PCR positive, further examinations led to a diagnosis of fungal endophthalmitis in ten patients. The fungal 28 S rDNA was detected in one non-infectious case (a false-positive case). In addition, there were two PCR false-negative cases that were clinically suspected of having fungal endophthalmitis.
Conclusions This novel quantitative broad-range PCR of fungal 28 S rDNA is a useful tool for diagnosing endophthalmitis related to fungal infections.

Keywords Polymerase chain reaction · Fungi · Ocular fluids · Endophthalmitis

Introduction

Fungal endophthalmitis can be caused by endogenous infections. These infections occur in patients who have systemic disorders (e.g., diabetes or malignancy), patients who use systemic drugs (e.g., broad-spectrum antibiotics, chemotherapeutic agents, or steroids), and patients who have intravascular catheters. In addition, fungal endophthalmitis can be caused by exogenous infections that arise from trauma or intraocular surgery. The clinical findings in some ocular infectious diseases caused by fungal species are quite diverse, with the exception of *Candida* infection. *Candida* infection in the eye is always characterized as endogenous endophthalmitis with fungal ball vitreous opacities. Moreover, fungal infections have been widely associated with various ocular disorders including endophthalmitis. Because of this diversity, it is often difficult to diagnose ocular fungal infections. Polymerase chain reaction (PCR) has been used to provide evidence of fungal involvement in suspected cases of intraocular infections. Previous studies have used PCR to demonstrate the presence of

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fungal DNA in the ocular fluids of patients with infectious endophthalmitis [1–3].

PCR-based methods make it possible to establish a diagnosis in less time than is required by standard cultures [4–6]. Moreover, studies have found that fungal cultures are negative in half of PCR-positive cases [1–3, 7]. The sensitivity of conventional culture techniques is not high, and these cultures take a long time due to their slow growth. Thus, the use of broad-range real-time PCR to analyze ocular samples may be a better way to obtain a rapid diagnosis in patients with unknown intraocular infectious diseases.

For the diagnosis of infectious endophthalmitis, broad-range real-time PCR for fungi is now available [1–3, 7]. To detect many types of fungal DNA, primers and probes for conserved regions in fungal sequences are used. We previously designed pan-fungal primers and probes that were complementary to the 18 S rRNA sequences present in the *Candida* and *Aspergillus* species, and we reported the efficacy of the technique for diagnosis [7]. This PCR technique detected all species of *Candida* and *Aspergillus* DNA. Although there were many advantages to using this PCR technique to diagnose fungal infection, there was one disadvantage. Although the fungal 18 S broad-range PCR detected *Candida* and *Aspergillus* DNA, it cannot detect other types of fungal DNA. Recently, a novel broad-range real-time PCR technique was developed for the rapid detection of human pathogenic fungi [8]. The assay targeted a part of the 28 S large subunit rRNA genes (28 S rDNA). Therefore, we prepared a new assay that targets a part of the 28 S rDNA found in species such as *Candida*, *Aspergillus*, *Cryptococcus*, *Trichophyton*, *Mucor*, *Penicillium*, and *Pichia*.

In the present study, we attempted to develop a novel fungal PCR examination that uses 28 S rDNA primers and the corresponding probes for the diagnosis of endophthalmitis related to fungal infection.

Materials and methods

Subjects

Based upon medical history and clinical observations, 497 patients (260 men and 237 women) were consecutively enrolled in a prospective study that was conducted at the Tokyo Medical and Dental University Hospital. The patient group consisted of patients with infectious endophthalmitis including clinically suspected bacterial and fungal endophthalmitis (n=76) and patients with infectious or non-infectious uveitis (n=421). The average patient age (±SD) was 60 (±16) years. After obtaining informed consent, samples of aqueous humor and vitreous fluids were collected from all patients.

In addition to the patient group, we also analyzed samples from a control group in which no patients had any type of ocular inflammation. The control patients were enrolled in this prospective study in 2009. Forty samples (20 aqueous humor samples and 20 vitreous fluid samples) were collected from the 40 control patients. The control group consisted of patients who had age-related cataract (n=20), macular edema (n=14), retinal detachment (n=4), idiopathic macular hole (n=1), and idiopathic epiretinal membrane (n=1).

For aseptic ocular sampling, the following procedures were performed in all subjects, as described in our previous reports [7, 9]. A 0.1-ml aliquot of aqueous humor was collected aseptically in a syringe with a 30-G needle. Half of the sample was then transferred into a pre-sterilized microfuge tube and used for PCR. In patients who were undergoing vitreous surgery, uncontaminated non-diluted vitreous fluid samples (0.5–1.0 ml) were collected during the diagnostic pars plana vitrectomy [7, 9]. Topical antibiotics were used in almost all patients before collecting samples, but oral antibiotics were not used.

The research followed the tenets of the Declaration of Helsinki, and all study protocols were approved by the Institutional Ethics Committee of Tokyo Medical and Dental University. The clinical trial was registered, and the information is available at www.umin.ac.jp/ctr/index/htm with study number R000002708. The study was started in July 2009 and was terminated in February 2011.

Quantitative polymerase chain reaction

DNA was extracted from the samples using a DNA Mini Kit (Qiagen, Valencia, CA, USA) installed on a Robotic workstation that was set for automated purification of nucleic acids (BioRobot EZ1 Advanced, Qiagen). The real-time PCR was performed by using an Amplitaq Gold and Light Cycler 480 II (Roche, Basel, Switzerland). Primers and probes of fungal 28 S rDNA are described elsewhere [8]. The sense primer was 5'-gcatatcaataagcggaggaaaag-3', and the antisense primer was 5'-ttagctttagatgRaRtttaccacc-3'. The probe (Dual-Labeled probe, Integrated DNA Technologies, Coralville, IA, USA) was 5'-FAM- cggcgagtgaagcgg-SaaRagctc–iowaBK-3'. Products were subjected to 50 cycles of PCR amplification, with cycling conditions set at 95 °C for 10 min, followed by 50 cycles at 95 °C for 0 s and 60 °C for 20 s. For PCR assay sensitivity, PCR fragments were amplified from the DNA of *C. albicans* (strain: ATCC 60193), *A. flavas* (strain: ATCC 22546), and *C. neoformans* (strain: ATCC 14116). The PCR results were obtained within 3 h after sample collection.

Amplification of the human β-globulin gene served as an internal positive extraction and amplification control. Fungal copy number values of more than 100 copies/ml in the sample were considered to be significant.

Results

Sensitivity of broad-range real-time PCR assay for fungal 28 S rDNA

To confirm the broad-range real-time PCR assay sensitivity, PCR fragments were amplified from the DNA of *Candida*, *Aspergillus*, and *Cryptococcus* species. The detection limit and standard range of the TaqMan real-time PCR were determined by using serial tenfold dilutions of linearized plasmid. The PCR results for the prepared samples showed that *C. albicans* DNA was detected at concentrations between 10^2 and 10^5 copies/ml (Fig. 1A). In addition, *Aspergillus* (Fig. 1B) and *Cryptococcus* DNA (Fig. 1C) were also detected at concentrations between 10^2 and 10^5 copies/ml. The best sensitivity for detecting *Candida*, *Aspergillus*, or *Cryptococcus* DNA was at a concentration of 10^1 copies/ml. No DNA was detected in the negative control (nuclease-free water).

Detection of fungal 28 S rDNA in suspected fungal endophthalmitis patients

The PCR results indicated that fungal 28 S rDNA was positive in 11 samples of ocular fluid from the endophthalmitis or uveitis patients (11/497, 2.2%). All 11 positive samples were detected in the infectious endophthalmitis patients (11/76, 14.5%). A representative PCR result in a case of endogenous endophthalmitis related to *Aspergillus* infection is shown in Fig. 2.

The PCR-positive patients had high copy numbers of fungal 28 S rDNA ranging from 1.7×10^3 to 7.9×10^6 copies/ml, which indicated the presence of fungal infection. Further examinations revealed that 10 of the 11 PCR-positive patients had fungal endophthalmitis; seven patients were diagnosed with endogenous endophthalmitis (3 *Candida,* 1 *Aspergillus*, 1 *Cryptococcus*, and 2 unknown), and three patients were diagnosed with late postoperative endophthalmitis (2 *Candida* and 1 unknown) (Table 1). Fungal 28 S rDNA was detected in only one non-infectious case (case 455 in Table 1). This PCR false-positive case had primary intraocular lymphoma that was diagnosed by monoclonal detection of B-cell IgH rearrangement by PCR, high amounts of IL-10 by ELISA, and detection of typical lymphoma cells (Class V) in the vitreous sample. Thus, fungal 28 S rDNA was detected in ocular samples from 10 patients with fungal endophthalmitis and one patient with non-infectious primary intraocular lymphoma.

However, two of the PCR-negative patients were clinically suspected to have fungal endophthalmitis (cases 24 and 461 in Table 1). PCR did not detect the fungal genome in the aqueous humor of these patients (<100 copies). *C. albicans* was detected in blood samples from case 461. Case 24 was a patient with endogenous endophthalmitis, and his blood tests were positive for β-D-glucan. Systemic antimycotic and topical antimycotic therapies were effective in the treatment of these two patients with false-negative results.

In conventional fungal cultures of ocular fluids, six (60%) of the 10 PCR-positive samples from fungal endophthalmitis patients were positive, and four samples were negative (Table 1). In addition, patients with fungicemia (cases 24, 179, 231, 326, 359, 461, and 490) had already begun therapy with antimycotic agents before PCR examinations (Table 2). Among these seven patients, six patients received intravenous hyperalimentation. On the other hand, patients without clinically apparent fungicemia (cases 30, 77, 161, and 355) initiated antimycotic drug therapy after receiving positive PCR results (Table 2).

The diagnostic parameters of sensitivity, specificity, positive predictive value, and negative predictive value of the PCR examinations for the diagnosis of fungal endophthalmitis were calculated to be 0.833, 0.998, 0.909, and 0.996, respectively.

Discussion

The broad-range real-time PCR assay amplified fungal 28 S rDNA in the ocular fluids of patients with clinically suspected fungal endophthalmitis. The 28 S rDNA of major fungal species for endophthalmitis, such as *Candida*, *Aspergillus*, and *Cryptococcus*, were detected by a novel broad-range real-time PCR examination ($>10^1$ copies/ml). Our fungal endophthalmitis patients were all immunocompetent, but almost all patients were older than 60 years of age, with the exception of two patients (cases 268 and 490). The PCR examination was negative for fungal DNA in cases of ocular inflammation caused by bacterial endophthalmitis or uveitis. In addition, fungal DNA was not detected in any of the 40 control patients without ocular inflammation.

Broad-range PCR for the 28 S rRNA sequence proved to be a reliable tool for the diagnosis of fungal endophthalmitis. Moreover, real-time quantitative PCR can be used to determine whether or not the fungus is related to the endophthalmitis. By using this PCR system, we were able to rapidly diagnose various types of fungal endophthalmitis in a few

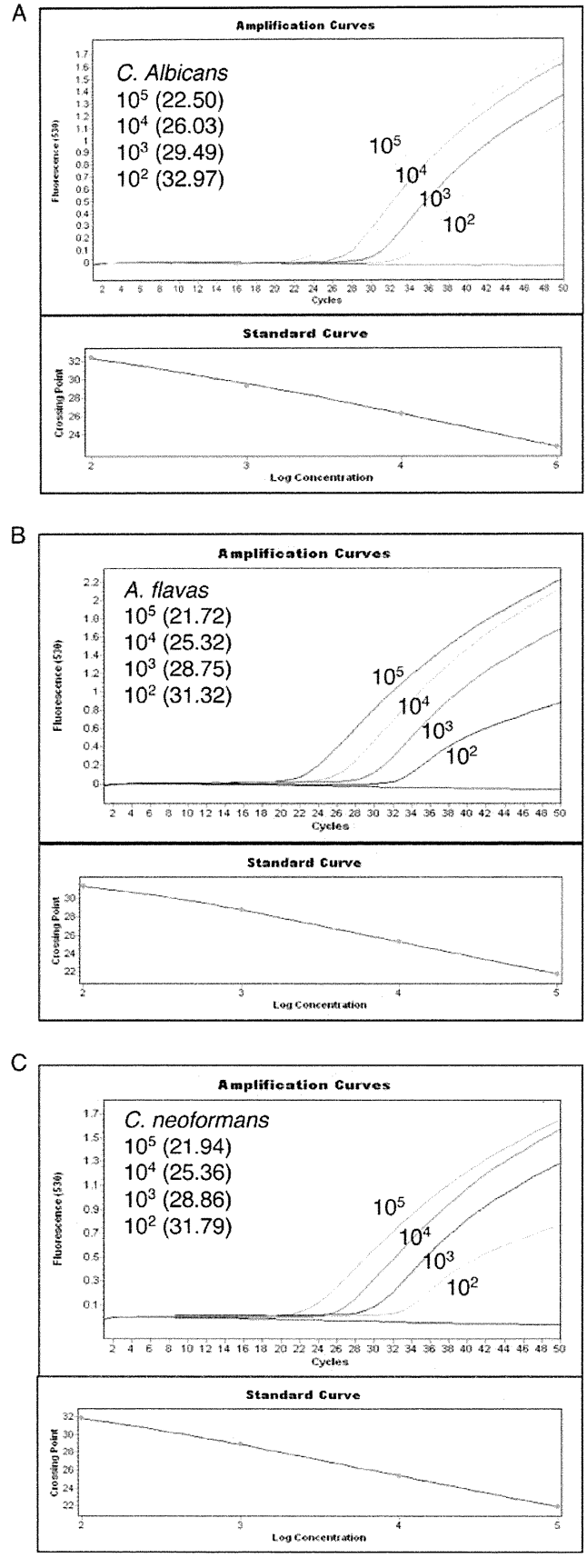

Fig. 1 PCR assay sensitivity. To examine the sensitivity of the broad-range real-time PCR for fungal 28 S rDNA, PCR fragments were amplified from DNA of *Candida* (*C. albicans,* **a**), *Aspergillus* (*A. flavas,* **b**), and *Cryptococcus* (*C. neoformans,* **c**) species. The *number in the parenthesis* indicates the cycle threshold (Ct) value in quantitative PCR

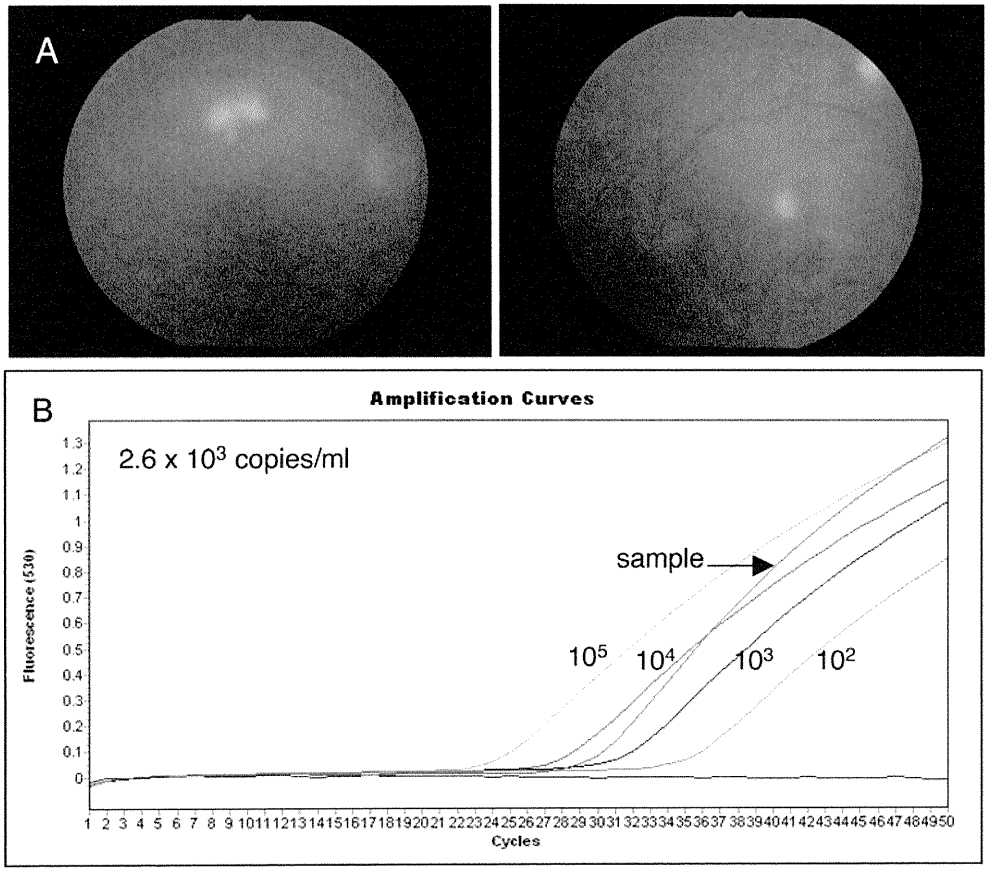

Fig. 2 A representative PCR result from a patient with endogenous endophthalmitis related to *Aspergillus* infection (case 179 in Table 1). A 74-year-old woman with type II diabetes mellitus was treated for splenic cancer. After surgery, she used intravascular catheters. She reported blurred vision and decreased visual acuity in her left eye. Ophthalmologic examination revealed characteristics of infectious endophthalmitis. **a** Fundus photographs of both eyes with a fungal infection. Retinal exudates together with retinal hemorrhages and slight vitreous opacity are seen. **b** Graph of the PCR results. The copy number of fungal genomic DNA in the sample was calculated. Real-time PCR for the fungal 28 S rDNA was performed with the ocular sample and the control DNA (10^5, 10^4, 10^3, and 10^2 copies/ml). We established the standard curve based on the results from the control DNA. Based on this standard curve, the sample Ct value was used to determine the DNA concentration of the sample. Final copy numbers of genomic DNA in the sample (copies/ml) were calculated based on the original sample volume and the final dilution volume. High copy numbers of fungal 28 S rDNA (2.6×10^3 copies/ml) were detected by real-time PCR

patients exhibiting clinical evidence of a fungal infection. In 40% of the PCR-positive patients, the fungal cultures that were performed on the same ocular fluid sample were negative. Thus, PCR-based methods make it possible to establish an etiologic diagnosis in less time than is required by standard cultures.

In addition, since these methods can detect very small numbers of DNA copies, they are extremely sensitive. These methods are also beneficial when used for intraocular infections, as only a relatively small volume of sample needs to be obtained at any one time. Since it is essential that treatments be started early in cases of infectious endophthalmitis, this broad-range real-time PCR system for ocular samples can provide a rapid diagnosis for patients who have an unknown intraocular disorder such as idiopathic uveitis or endophthalmitis. Additionally, when minimal amounts of ocular samples are available, it is difficult to perform a culture test to detect fungi [4–6]. Therefore, the use of PCR to detect the fungal genome in ocular fluids is advantageous.

We previously developed a novel PCR assay to detect fungal infection by amplifying fungal 18 S rRNA genes [7]. The broad-range real-time PCR detected a few *Candida* species (*C. albicans, C. parapsilosis, C. tropicalis, C. guilliermondii, C. glabrata,* and *C. krusei*), along with *Aspergillus* species (*A. fumigatus, A. flavus, A. nidulans, A. niger,* and *A. terreus*). By using several different primers and probes, we were able to separately detect each of these fungal species. *Candida* or *Aspergillus* DNA was detected in seven of 54 ocular samples (13%) from patients with unknown uveitis or endophthalmitis. These PCR-positive samples showed significantly high copy numbers of *Candida* or *Aspergillus* DNA. On the other hand, fungal DNA was not detected in the other 46 samples collected from these

Table 1 Detection of fungal 28 S rDNA in endophthalmitis and uveitis patients

Case no.	Age/ gender	Diagnosis	Sample	DNA (ng/ml)	Real-time PCR (copies/ml)	Cultures with ocular fluids	Fungal blood test[a]
24	76/M	Endogenous endophthalmitis	AH	26.1	$<10^2$	Negative	BDG - 30.2
30	67/F	Late postoperative endophthalmitis	AH	21.4	2.0×10^4	*Candida albicans*	BDG - negative
77	85/M	Endogenous endophthalmitis	VF	41.3	7.6×10^3	*Candida* spp.	nt
161	75/M	Endogenous endophthalmitis	VF	57.8	2.8×10^5	*Candida albicans*	nt
179	74/F	Endogenous endophthalmitis	AH	29.1	2.6×10^3	Negative	*Aspergillus* antigen - 36.8
231	64/M	Endogenous endophthalmitis	VF	105	1.7×10^3	Negative	BDG - 11.9
268	40/M	Endogenous endophthalmitis	VF	30.2	2.2×10^5	*Cryptococcus neoformans*	BDG - negative
326	86/F	Endogenous endophthalmitis	VF	85.7	1.5×10^5	Negative	BDG - 51.7
355	81/M	Late postoperative endophthalmitis	AH	62.7	7.9×10^6	nt	nt
359	69/M	Late postoperative endophthalmitis	VF	20	5.1×10^4	*Candida* spp.	BDG - 36.8
455	67/M	Primary intraocular lymphoma	VF	41.4	5.0×10^4	nt	nt
461	63/M	Endogenous endophthalmitis	AH	35.7	$<10^2$	Negative	BDG - 24.6; *C. acbicans*
490	49/M	Endogenous endophthalmitis	AH	43	6.6×10^4	*Candida albicans*	BDG - 449; C. acbicans

Using broad-range quantitative PCR, fungal 28 S rRNA gene (rDNA) could be detected in 11 ocular samples of ten fungal endophthalmitis cases and one non-infectious case

AH aqueous humor, *nt* not tested, *PPV* pars plana vitrectomy, *SA* systemic antimycotic (oral or intravenous), *TA* topical antimycotic, *VF* vitreous fluids

[a] Fungal blood test levels of β-D-glucan (BDG: pg/ml), detection of fungal antigens (pg/ml), and conventional fungal cultures

idiopathic uveitis or endophthalmitis patients [7]. However, this PCR examination could not detect other types of fungal infections. Therefore, as the next step, we have developed a novel PCR examination for broad fungi diagnosis. We attempted to detect whole-genomic fungal DNA in humans by PCR amplification of 28 S rDNA [8].

The new assay targets a part of the 28 S rDNA found in *Candida*, *Aspergillus*, *Cryptococcus*, *Mucor*, *Penicillium*, *Pichia*, *Microsporum*, *Trichophyton*, and *Scopulariopsis* [8]. It is assumed that infectious endophthalmitis related to fungal infection may be caused by various human pathogenic fungi, and the ocular infection may indicate various types of endophthalmitis, such as endogenous, post-

Table 2 Summary of risk factors and therapies in fungal endophthalmitis patients

Case no.	Diagnosis	Risk factors	A	B	C	Outcome
24	Endogenous endophthalmitis	IVH, peritoneal catheter	(+)	SA	PPV, SA	Resolved
30	Late postoperative endophthalmitis	Vitrectomy, IOL second implant	(-)	None	PPV, SA	Resolved
77	Endogenous endophthalmitis	Diabetes	(-)	PPV	SA	Resolved
161	Endogenous endophthalmitis	Diabetes	(-)	PPV	Unknown	Unknown
179	Endogenous endophthalmitis	Diabetes, pancreas carcinoma, IVH	(+)	SA, TA	SA, TA	Resolved
231	Endogenous endophthalmitis	Gallbladder carcinoma, IVH	(+)	PPV, SA	SA	Resolved
268	Endogenous endophthalmitis	Triamcinolone subtenon injection	(-)	PPV	SA	Resolved
326	Endogenous endophthalmitis	Aortocoronary bypass, IVH	(+)	PPV, SA	SA, TA	Resolved
355	Late postoperative endophthalmitis	PEA + IOL	(-)	None	TA	Resolved
359	Late postoperative endophthalmitis	PEA + IOL	(+)	PPV, SA	SA	Resolved
455	Primary intraocular lymphom	Lymphoma, steroid use	(-)	Methylprednisolone	Methotrexate	Resolved
461	Endogenous endophthalmitis	Aortocoronary bypass, IVH	(+)	SA	SA	Resolved
490	Endogenous endophthalmitis	Subarachnoid hemorrhage, IVH	(+)	PPV, SA	SA, TA	Resolved

A = presence or absence of antimycotic therapy before PCR examination

B = therapy prior to PCR examination

C = therapy after PCR examination

IVH intravenous hyperalimentation, *IOL* intraocular lens, *PEA* phacoemulsification and aspiration, *PPV* pars plana vitrectomy, *SA* systemic antimycotic (oral or intravenous)

traumatic, post-operative, and ocular surface infection (e.g., corneal ulcer). Furthermore, real-time PCR assays play an important role among molecular genetic screening methods because of the rapid diagnostic outcome. As shown in the current study, a broad-range real-time PCR assay targeting clinically relevant fungal species in one assay is now available.

In two false-negative cases that were clinically suspected of having fungal endophthalmitis (perhaps *Candida*-associated), our fungal 28 S PCR did not detect any fungal genome in the ocular samples. However, it should be noted that these samples were aqueous humor and not vitreous fluid. If a vitreous sample or a retinal tissue sample had been obtained by biopsy, we might have detected fungal DNA by this PCR method, because endophthalmitis, especially *Candida* spp., often results from hematogenous dissemination. In cases of postoperative endophthalmitis related to fungal infection, the result from an aqueous humor sample as well as a vitreous sample may be reliable. In fact, two aqueous humor samples were used to detect late postoperative endophthalmitis in the current study. Thus, the type of sample that is collected may be very important for an accurate diagnosis. Depending on the exact clinical setting, a vitreous sample likely offers an optimal diagnosis, since the cultures are usually more accurate. An aqueous sample is, obviously, more easily accessible, but the diagnostic power should be quoted only for the type of sample so as not to confuse expectations and dependency on the results. In cases of fungal endophthalmitis in immunocompetent patients, specific additional antimycotic therapy has been shown to be effective in controlling the ocular inflammation [10–12]. In fact, our PCR-negative immunocompetent patients were finally well controlled by the antimycotic treatment.

In conclusion, we developed a novel protocol for the rapid detection of fungal DNA in ocular samples that was based on fungal species that commonly cause eye disorders. This broad-range real-time PCR method can be utilized for rapid diagnosis of patients who have unknown infectious intraocular disorders. For clinicians to be able to identify the type of fungi, we may need to consider the use of sequence analysis. In the near future, we may be able to determine the fungal species via sequence analysis and rapidly diagnose fungal endophthalmitis; then, we will be able to promptly begin appropriate treatment with antimycotic drugs.

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Competing interests None.

Contributors MO was the principal investigator, designed and performed experiments, and wrote the manuscript. SS designed and conceptualized the study and drafted and edited the manuscript. KW and NS performed PCR assays. MM designed and conceptualized the study and edited the manuscript.

Data sharing statement No additional data.

Ethics approval Ethics approval was provided by the Institutional Ethics Committee of Tokyo Medical and Dental University.

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INFLAMMATORY DISORDERS

Detection of *Candida* and *Aspergillus* species DNA using broad-range real-time PCR for fungal endophthalmitis

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Abstract
Background The goal of this work is to establish a broad-range real-time polymerase chain reaction (PCR) diagnostic system for ocular fungal infection and to measure *Candida* and *Aspergillus* DNA in the ocular fluids obtained from unknown uveitis/endophthalmitis patients.
Methods After obtaining informed consent, intraocular fluids (aqueous humor and vitreous fluid samples) were collected from 54 patients with idiopathic uveitis or endophthalmitis. Samples were assayed for *Candida* or *Aspergillus* DNA using broad-range (18S rRNA sequences) quantitative real-time PCR.
Results *Candida* or *Aspergillus* DNA was detected in seven out of 54 patient ocular samples (13%). These PCR-positive samples showed significantly high copy numbers of *Candida* or *Aspergillus* DNA. On the other hand, fungal DNA was not detected in any of the other 46 samples collected from these idiopathic uveitis or endophthalmitis patients. In the one PCR-negative case, PCR did not detect any fungal genome in the sample, even though this patient was clinically suspected of having *Candida* endophthalmitis. Real-time PCR results were negative for fungal DNA in the bacterial endophthalmitis patients and in various uveitis patients. In addition, fungal DNA was also not detected in patients without ocular inflammation (controls).
Conclusions Analysis of ocular samples by this broad-range real-time PCR method can be utilized for rapid diagnosis of patients suffering from unknown intraocular disorders such as idiopathic uveitis/endophthalmitis.

Keywords Endophthalmitis · Fungal infection · Polymerase chain reaction

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Introduction

Fungal endophthalmitis is a sight-threatening disease caused by human pathogenic fungi. Fungal infections are known to cause ocular inflammations such as endophthalmitis, uveitis, and keratitis. However, with the exception of for the *Candida*-associated ocular infection, the association between the fungus and the observed clinical features has yet to be elucidated. The well-known clinical features for *Candida* endophthalmitis include a fungal ball in the retina and vitreous opacity [1]. Fungal endophthalmitis can result from hematogenous dissemination or from a direct inoculation following trauma or surgery to the eye. Risk factors for fungal endophthalmitis include intravascular catheters, diabetes, malignancy, chemotherapeutic agents, and steroids. However, the clinical findings can be very diverse in some cases of ocular inflammatory disorders caused by fungal species. Moreover, fungal infections have been widely associated with keratitis, retinitis, uveitis, retinal/choroidal vasculitis, invasive orbital infection, and endophthalmitis. Because of this diversity, infection diagnosis is both difficult and time-consuming [1–4]. In order to be able to perform adequate treatments that can prevent these infectious agents from causing irreversible ocular damage,

early examinations that correctly identify the etiology of the infection are necessary.

Conventional methods of diagnosis of fungal endophthalmitis include detection and isolation of the fungi from the intraocular fluids (aqueous humor or vitreous). However, since the sensitivity of conventional fungal cultures is not high, and the culture growth rates are slow, longer times are required before final results can be obtained [5, 6]. Thus, an early diagnosis can be important in ensuring there is prompt management of the endophthalmitis. Previous studies have shown that polymerase chain reaction (PCR) can be successfully and reliably used to make a diagnosis of fungal endophthalmitis [7–10]. However, even conventional PCR has yet to be able to determine quantitative information for the fungal genome in ocular samples.

In this study, we used real-time quantitative PCR for detection of *Candida* and *Aspergillus* DNA. We developed a protocol for the rapid detection of fungal DNA in ocular samples that was based on two major species (*Candida* and *Aspergillus*) that commonly cause eye disorders. We designed novel panfungal primers and probes that were complementary to the 18S rRNA sequences present in these species. Our broad-range real-time PCR proved to be an accurate method for quantitating fungal copies of both *Candida* and *Aspergillus* DNA.

Methods

Sample preparation

From 2006 to 2010, we consecutively enrolled endophthalmitis and uveitis patients in a prospective study that was conducted at our hospital (Table 1). After informed consent was obtained in all patients, we collected aqueous humor and vitreous fluid samples. A 0.1–0.2 ml aliquot of aqueous humor (asepsis) was collected in a syringe with a 30-G needle. We also collected non-diluted vitreous fluid samples (0.5–1.0 ml) during diagnostic pars plana vitrectomy (PPV) procedures that were conducted in patients with clinically suspected fungal endophthalmitis/uveitis. All of the patients displayed active intraocular inflammation at the time of sampling. The samples were transferred into a pre-sterilized microfuge tube and used for PCR. To ensure that no contamination of the PCR preparation occurred, the DNA amplification and the analysis of the amplified products were done in separate laboratories, as per a method reported for one of our previous studies [11].

For cultures of fungi, the Bacteria Work Station of the Tokyo Medical and Dental University Hospital processed all specimens (aqueous humor and vitreous fluids) within 1 h after the sample collection, with standard methods followed for the isolation and identification of fungal cultures [11].

In addition to the patient groups, we also analyzed samples from a control group. A total of 40 samples (20 aqueous humor and 20 vitreous fluids) were collected from patients who did not have any type of ocular inflammation (age-related cataract, macular edema, retinal detachment, idiopathic macular hole, or idiopathic epiretinal membrane).

The research followed the tenets of the Declaration of Helsinki and all study protocols were approved by the Institutional Ethics Committee of Tokyo Medical and Dental University. This clinical trial was registered, with registration information available at www.umin.ac.jp/ctr/index/htm. The study number attached to this registration is R000002708. The study was begun in April of 2006 and ended in April of 2010.

Polymerase chain reaction

To detect the *Candida* and *Aspergillus* DNA, we designed primers and probes for the broad-range PCR of the 18S rRNA sequences, which we have described in a previous report [10]. Kami et al. [12] developed primers and a probe for real-time PCR and demonstrated that the procedure was highly specific for the *Aspergillus* infection. In this study, we also designed a probe for use in the *Candida* species DNA amplifications (Fig. 1).

DNA was extracted from the samples using a DNA Mini Kit (Qiagen, Valencia, CA) installed on a robotic workstation that was set for automated purification of nucleic acids (BioRobot E21, Qiagen). The real-time PCR was performed using the Amplitaq Gold and the Real-Time PCR 7300 system (Applied Biosystems, Foster City, CA) or Light Cycler 480 II (Roche, Switzerland). The paired primers and TaqMan probes used for *Candida* and *Aspergillus* are shown in Fig. 1. Products were subjected to 50 cycles of PCR amplification, with cycling conditions set at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. For PCR assay sensitivity, PCR fragments were amplified from the DNA of *C. albicans* (Strain: ATCC 60193). Amplification of the human β-globulin gene served as an internal positive extraction and amplification control. Copy number values of more than ten copies/ml in the sample were considered to be significant.

Results

Specificity of *Candida* and *Aspergillus* species in broad-range real-time PCR

To evaluate the specificity of the *Candida* and *Aspergillus* species using broad-range real-time PCR of the 18S rRNA sequences, total nucleic acids of six *Candida* species and five *Aspergillus* species were extracted and assayed for 18S

Table 1 Detection of *Candida* and *Aspergillus* 18S rRNA gene by broad-range real-time PCR in unknown uveitis or endophthalmitis patients and control uveitis patients

Initial diagnosis	No. of patients	Sample	Results for real-time PCR	Final diagnosis	Remarks
Idiopathic uveitis/ endophthalmitis	n=46	Aqh, VF	<10 copies	Idiopathic uveitis/ endophthalmitis	
	n=1 (65, male)	VF	*Candida* 9.2×10^5 copies/ml	*Candida* endophthalmitis	Case 1; Endogenous endophthalmitis
	n=1 (71, female)	VF	*Aspergillus* 4.5×10^2 copies/ml	*Aspergillus* endophthalmitis	Case 2; Endogenous endophthalmitis
	n=1 (73, male)	VF	*Aspergillus* 1.8×10^3 copies/ml	*Aspergillus* endophthalmitis	Case 3; Late postoperative endophthalmitis
	n=1 (80, male)	Aqh	*Candida* 3.4×10^2 copies/ml	*Candida* endophthalmitis	Case 4; Post-traumatic corneal ulceration
	n=1 (66, female)	VF	*Candida* 6.5×10^5 copies/ml	*Candida* endophthalmitis	Case 5; Endogenous endophthalmitis (IFN treatment)
	n=1 (74, male)	VF	*Candida* 6.2×10^4 copies/ml	*Candida* endophthalmitis	Case 6; Endogenous endophthalmitis (diabetes)
	n=1 (0, female)	VF	*Candida* 9.4×10^4 copies/ml	*Candida* endophthalmitis	Case 7; Endogenous endophthalmitis (normal infant)
	n=1 (60, male)	Aqh	<10 copies	*Candida* endophthalmitis	Case 8; Endogenous endophthalmitis (IVH use)
Bacterial endophthalmitis	n=7	Aqh, VF	<10 copies	/	
Sarcoidosis	n=4	Aqh, VF	<10 copies	/	
Vogt-Koyanagi-Harada disease	n=1	Aqh	<10 copies	/	
Toxocariasis	n=1	Aqh	<10 copies	/	
Toxoplasmosis	n=3	Aqh, VF	<10 copies	/	
Acute retinal necrosis	n=7	Aqh, VF	<10 copies	/	
Cytomegalovirus retinitis	n=4	Aqh, VF	<10 copies	/	
Herpetic anterior iridocyclitis	n=4	Aqh	<10 copies	/	
Non-inflammatory ocular diseases*	n=40	Aqh, VF	<10 copies	/	Controls for PCR

*Non-inflammatory ocular diseases: age-related cataract, macular edema, retinal detachment, idiopathic macular hole or idiopathic epiretinal membrane

Aqh aqueous humor, *IFN* interferon, *IVH* Intravenous hyperalimentation, *VF* vitreous fluids

rDNA. As seen in Fig. 1, the broad-range real-time PCR detected six *Candida* species, i.e., *C. albicans, C. parapsilosis, C. tropicalis, C. guilliermondii, C. glabrata,* and *C. krusei*, along with five *Aspergillus* species, i.e., *A. fumigatus, A. flavus, A. nidulans, A. niger,* and *A. terreus*. By using several different primers and probes, we were able to separately detect each of these fungal species (Fig. 1).

Sensitivity of the real-time PCR assay

To confirm the broad-range real-time PCR assay sensitivity, PCR fragments were amplified from the DNA of *C. albicans*. The detection limit and standard range of the TaqMan real-time PCR were determined by using serial tenfold dilutions of linearized plasmid. The PCR results for the prepared samples showed that the best sensitivity for detecting *C. albicans* DNA was at a concentration of 10^1 per PCR (Fig. 2). There was no detection of the DNA in the negative control (nuclease-free water).

Detection of *Candida* and *Aspergillus* 18S rRNA gene in unknown uveitis/endophthalmitis patients

PCR results indicated a total of seven ocular fluid samples from the idiopathic uveitis or endophthalmitis patients (7/54, 13% positive, Table 1) were positive for *Candida* or *Aspergillus* DNA. These positive patients had high copy numbers of either *Candida* or *Aspergillus* DNA, with values ranging from 3.4×10^2 to 9.2×10^5 copies/ml. These results indicate the presence of a fungal infection. A representative PCR result is shown in Fig. 3. Conversely, conventional fungal cultures only found two out of the seven PCR-positive samples (both *C. albicans*) to be positive, while the other five samples were negative.

On the other hand, fungal DNA was not detected in any of the other 46 samples collected from these idiopathic uveitis or endophthalmitis patients. In the one PCR-negative case, PCR did not detect any fungal genome in the aqueous humor (<10 copies, case 8 in Table 1), even

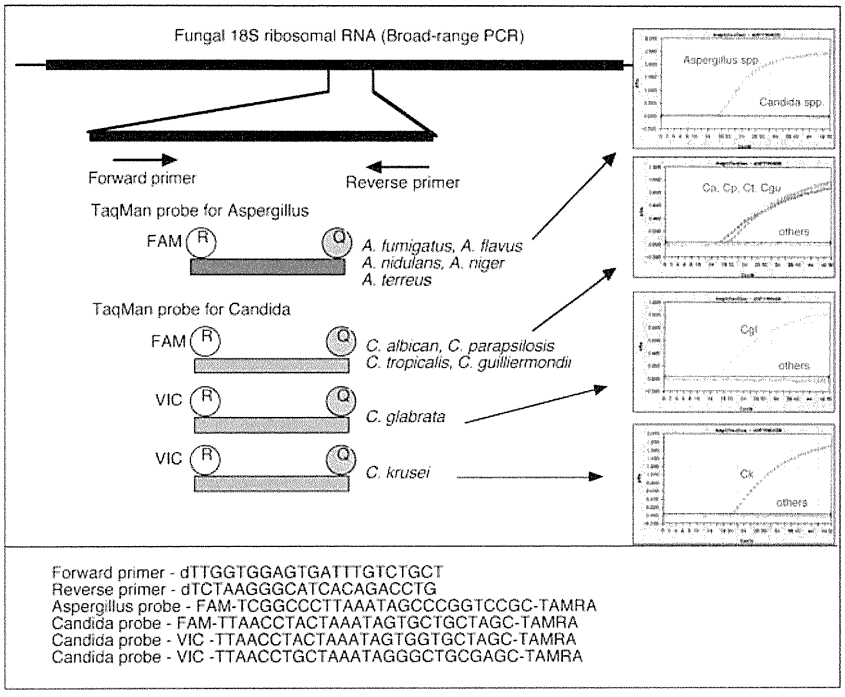

Fig. 1 Specific primers and probes for broad-range real-time PCR of the fungal 18S rRNA sequence were designed in order to detect DNA for *Candida* and *Aspergillus* species

though this patient was clinically suspected of having *Candida* endophthalmitis. Real-time PCR results were negative for the *Candida* and *Aspergillus* DNA in the bacterial endophthalmitis patients (n=7) and in the various uveitis patients (n=24) who had been diagnosed with sarcoidosis, Vogt-Koyanagi-Harada disease, toxocariasis, toxoplasmosis, acute retinal necrosis, cytomegalovirus retinitis, or herpetic anterior iridocyclitis. In addition, fungal DNA was not detected in any of the 40 control samples that were collected from the patients without ocular inflammation.

Of the seven patients who were PCR positive, further examinations led to fungal endophthalmitis diagnoses as follows: five patients had endogenous endophthalmitis (four *Candida* and one *Aspergillus*), one had late postoperative endophthalmitis (*Aspergillus*, case 3), and one had post-traumatic keratitis-associated endophthalmitis (*Candida*, case 4) (Table 1).

Case reports

Case 1

A 65-year-old man with type II diabetes mellitus was treated for unknown uveitis over a period of a few weeks during 2009. He complained of blurred vision, decreased visual acuity, and pain in his right eye (RE). Ophthalmologic examination demonstrated the presence of characteristics of uveitis, bacterial endophthalmitis and fungal endophthalmitis. Vitreous opacity, including the presence of a fungal ball and yellowish retinal exudates, was seen in the fundus of his RE (Fig. 4a). After vitrectomy of his RE,

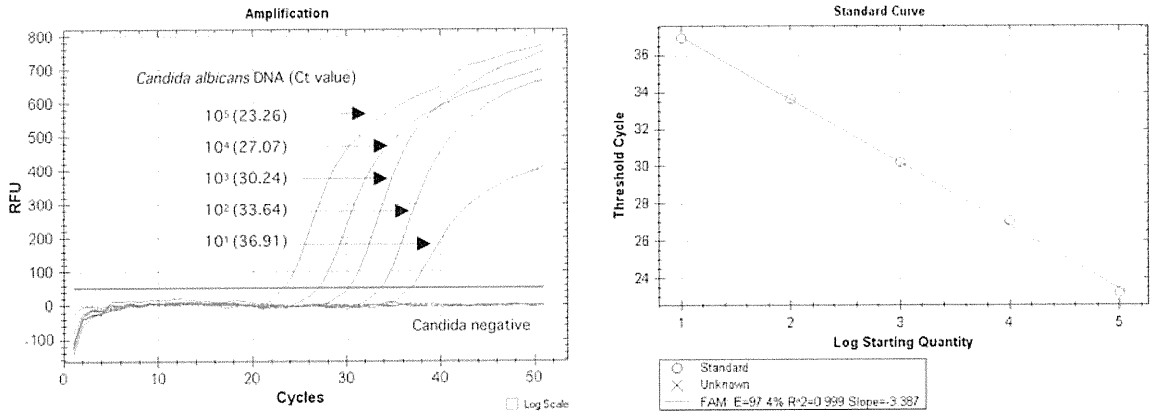

Fig. 2 In order to examine broad-range real-time PCR assay sensitivity for the fungal 18S PCR, the PCR fragments were amplified from the DNA of *C. albicans* (ATCC 60193). The *number in parenthesis* indicates the cycle threshold (Ct) value in quantitative PCR

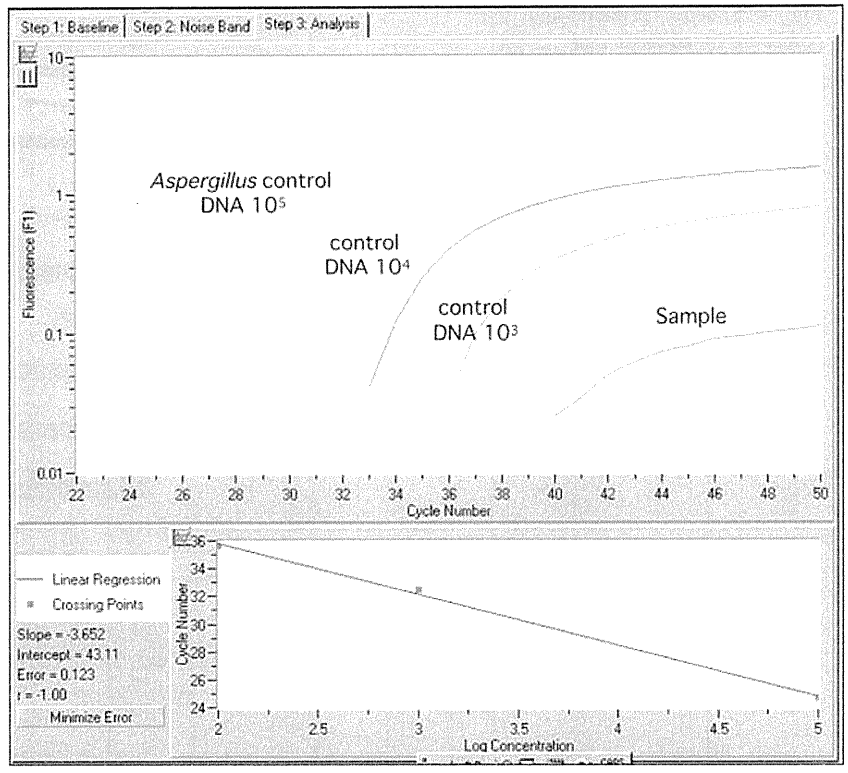

Fig. 3 Representative data for the broad-range real-time PCR. *Aspergillus* DNA (4.5×10^2 copies/ml) but not *Candida* DNA was detected in the vitreous sample of case 2

real-time PCR of the vitreous sample obtained during the procedure indicated there were high copy numbers of *Candida* DNA (9.2×10^5 copies/ml, Fig. 4b). Based on these results, the patient was given systemic fluconazole (Table 1). *Aspergillus* DNA was not detected in this sample. A few days later, fungal culture of his vitreous specimen

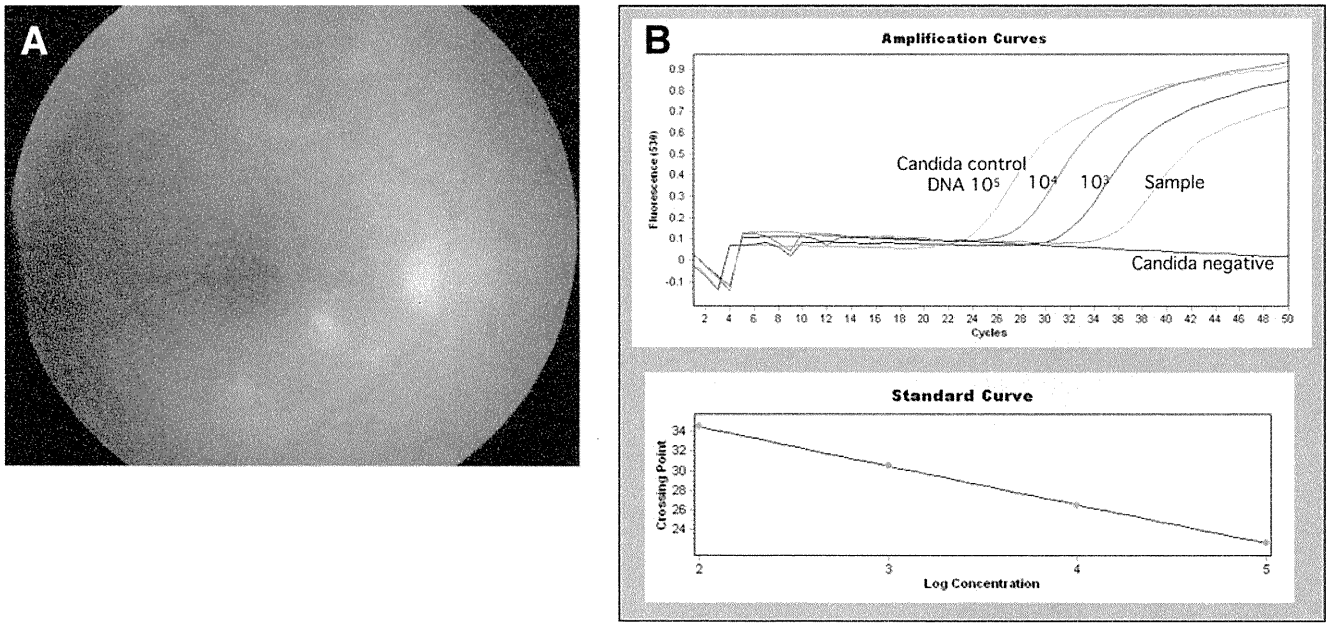

Fig. 4 PCR results for case 1. **a** Fundus photograph of the right eye with a *Candida* infection. Dense vitreous opacity and retinal exudates are seen. **b** This is a graph of the PCR results. We calculated the copy number of fungal genomic DNA in the sample. After we measured both the tested ocular sample and the control DNA (10^5, 10^4, and 10^3 copies/ml) using real-time PCR, we then established the standard curve based on the results of the control DNA. Based on this standard curve, the sample Ct value was used to determine the DNA concentration of the sample. Final copy numbers of genomic DNA in the sample (copies/ml) were calculated based on the obtained sample volume and final dilution volume. High copy numbers of *Candida* DNA (9.2×10^5 copies/ml) were detected by PCR. *Aspergillus* DNA was not detected in the sample

was also found to be positive for *C. albicans*. After being treated, he had complete resolution of his symptoms.

Case 3

A 73-year-old man was referred to the Uveitis Clinic at our hospital in July 2008 because of keratic precipitates (KPs), cells in the anterior chamber, and anterior vitreous opacity in his RE that was associated with recurrent anterior uveitis. In his RE, diffuse pigmented KPs were seen (Fig. 5a). After considering both the clinical features and whole body inspections, we diagnosed this case as idiopathic uveitis. Although he was treated with topical corticosteroid and an antibiotic for 2 months, the KPs expanded (Fig. 5b). During the treatment, diffuse pigmented KPs continued to expand and then united. In addition, we also observed cells in the anterior chamber with hypopyon and dense anterior vitreous opacity. After informed consent was obtained, pars plana vitrectomy was performed in order to obtain a vitreous sample. Although fungi were not detected in a culture test, real-time PCR detected 1.8×10^3 copies/ml of the *Aspergillus* 18S rRNA gene (Table 1). Microbiological investigations performed using both culture and Gram's staining of the vitreous sample proved to be negative. A blood test for β-D-glucan and fungal antigens including *Aspergillus* were also negative. We diagnosed the patient as having *Aspergillus*-associated late postoperative endophthalmitis that was related to his 2007 cataract surgery. The patient was subsequently treated using systemic fluconazole. The medication proved to be effective in treating the infectious endophthalmitis, with the inflammation in the anterior segment of his RE completely disappearing (Fig. 5c). After treatment, *Aspergillus* DNA in his sample was below the PCR detection level.

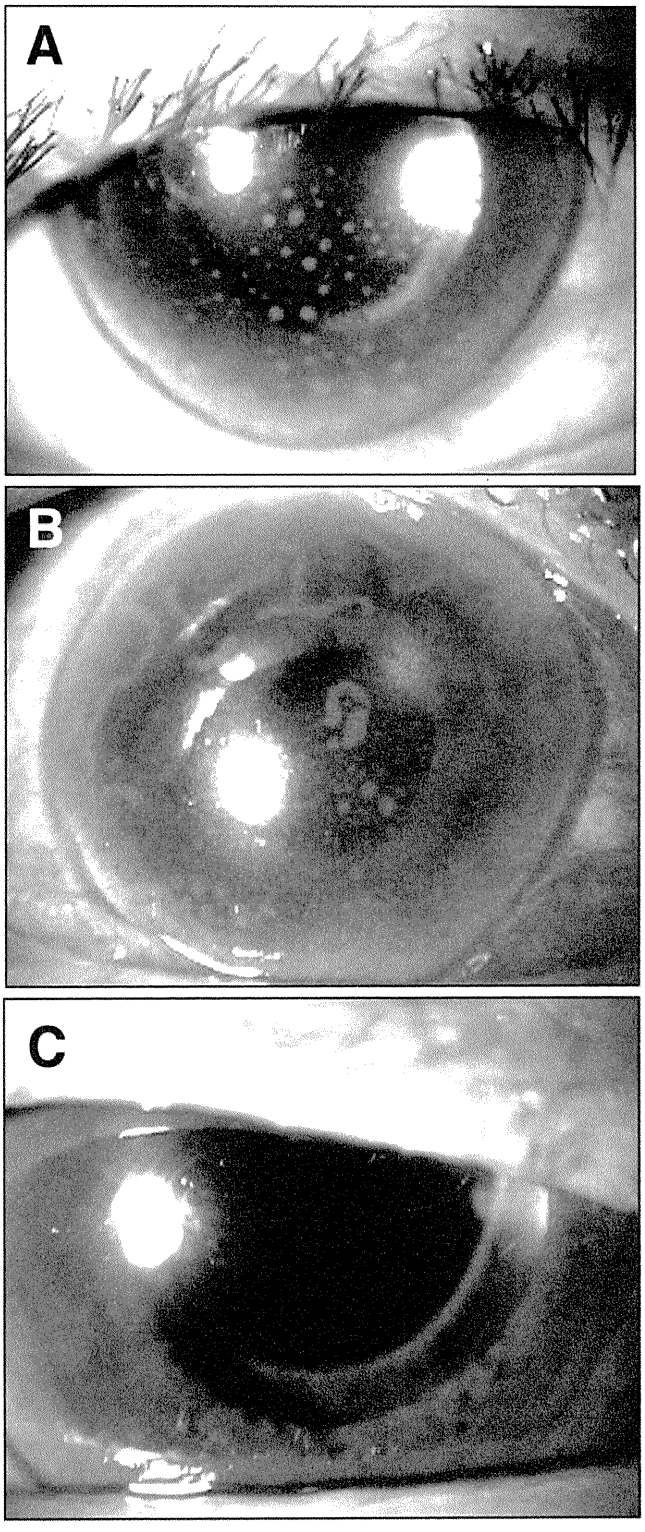

Fig. 5 PCR results for case 3. **a** Slit photograph of the right eye with an *Aspergillus* infection. Diffuse pigmented keratic precipitates (KPs) are seen. **b** The pigmented KPs are expanded and united. Like the previous case, the *Aspergillus* DNA gene (1.8×10^3 copies/ml) but not the *Candida* DNA was detected in the sample. **c** After treatment, the inflammation completely disappeared

Discussion

PCR is well suited for the detection of fungal moieties due to its specificity and applicability for use with small samples such as ocular specimens. Moreover, real-time quantitative PCR can be used to determine whether or not the fungus is related to endophthalmitis. By utilizing our broad-range real-time PCR for the 18S rRNA sequence, we were able to rapidly diagnose *Candida* or *Aspergillus* endophthalmitis in a few patients that exhibited clinical evidence of a fungal infection. While our methodology showed both positive and negative results, it was generally more helpful than waiting for culture results, as the culture tests used to detect *Candida* or *Aspergillus* are both difficult to perform and require longer amounts of time due to the slow growth rates for these species [5, 6, 13]. In addition, the specificity of our PCR examination is good enough so

that even a negative test is of benefit, as it helps to prevent making an incorrect diagnosis and administering a treatment for an infectious agent that is not present. Thus, this broad-range and real-time PCR system for ocular samples can provide a rapid diagnosis for those patients suffering from an unknown intraocular disorder such as idiopathic uveitis or endophthalmitis.

Fungal endophthalmitis is a sight-threatening disease that is most commonly caused by the *Candida* species. This disease usually accounts for a few percent of all of the cases of culture-proven endophthalmitis. The disease is normally acquired from an endogenous source that is spread by hematogenous dissemination. However, its occurrence may also be secondary to trauma, intraocular surgery, or corneal ulceration.

As confirmation of this suspected clinical disease is often difficult, there is frequently a delay in starting treatments. In the present patients, it was difficult to ascertain whether *Candida* or *Aspergillus* species were the causative agent in the intraocular inflammation. Since, in general, all of the patients were elderly and were immunocompetent, there was no focus area for the fungal infection systemically. As seen in Table 1, however, there were three exceptions. These included one case with a history of trauma (case 4), one case with a history of ocular surgery (case 3), and one case involving a normal infant (case 7), and for whom the case report details have been previously published [14].

In cases of fungal endophthalmitis in immunocompetent patients, specific additional antimycotic therapy has been shown to be effective in controlling the inflammation in the eye. In fact, all of the patients who were rapidly diagnosed by this PCR method were well controlled by the antimycotic treatment. Moreover, our PCR system was not only able to detect the conserved sequence of the fungal 18S rRNA gene, but it was also able to provide quantitative information from the ocular samples.

In recent years, PCR technology has been demonstrated to have a great potential in the detection and identification of low copy numbers of a microorganism's DNA in clinical samples [7–12, 15, 16]. It also holds great promise for being able to identify small numbers of organisms in small sample volumes, a situation that is commonly seen when trying to examine intraocular samples from patients with infectious endophthalmitis. We evaluated these PCR techniques in order to determine a reliable and effective protocol for detecting *Candida* or *Aspergillus* species DNA in ocular samples. Our specific aims were to try and significantly increase the number of intraocular samples from which a confirmed diagnosis could be made and to reduce the time it took to make a mycologic diagnosis. In many previous reports, DNAs of *Candida* and *Aspergillus* species were detected in patients with clinically suspected fungal endophthalmitis [7–10, 15–20]. For example, *Candida* species such as *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. glabrata*, and *C. krusei* have been increasingly recognized as being capable of causing fungal endophthalmitis. However, *C. albicans* has been shown to be the causative agent in the majority of cases of culture-proven endophthalmitis. Moreover, *Aspergillus* such as *A. fumigatus*, *A. flavus, A. nidulans*, *A. niger,* and *A. terreus* have also been reported to be the causative species in an unknown ocular infection [17–20]. To detect these fungal species, our present PCR system used paired primers and specific probes that were based upon the 18S rRNA genes of *Candida* and *Aspergillus* (see Fig. 1).

In one patient who was clinically suspected of having *Candida* endophthalmitis, our new PCR method did not detect any fungal genome in the ocular sample (case 8 in Table 1). However, it should be noted that this sample was aqueous humor and not vitreous fluid. Perhaps if a vitreous sample had been obtained, we might have detected *Candida* DNA, as *Candida* endophthalmitis often results from hematogenous dissemination. In fact, this particular patient received intravascular catheters after his initial surgery. Thus, in order to be able to make an accurate diagnosis, the type of sample that is collected may be very important.

Although there are many advantages for using our PCR assay, there is one disadvantage when attempting to diagnose fungal ocular infection. While our PCR examination was able to detect all species of *Candida* and *Aspergillus* DNA, it could not detect other fungi DNA. Recently, Vollmer et al. reported on a novel broad-range real-time PCR assay for the rapid detection of human pathogenic fungi [21]. Their assay targeted a part of the 28S large subunit rRNA (rDNA) gene. Since this PCR assay can examine *Candida* species, *Aspergillus* species, *Cryptococcus* species, among others, we are currently trying to develop a new PCR examination that uses these primers and probes for the diagnosis of fungal ocular infections, including fungal endophthalmitis.

In conclusion, utilization of the PCR assay to examine ocular samples in patients with suspected fungal endophthalmitis and idiopathic uveitis or endophthalmitis appears to be clinically useful for detecting *Candida* and *Aspergillus* DNA. Thus, broad-range PCR for the 18S rRNA sequence is a reliable tool for the diagnosis of fungal endophthalmitis and in screening for fungal infections. Moreover, because real-time PCR is an accurate method of quantitating fungal copies, real-time quantitative PCR can be used to determine whether the fungus is related to the endophthalmitis. Since the sensitivity of conventional culture techniques is not high and these cultures tend to take a long time due to their slow growth, the use of a broad-range and real-time PCR system to analyze ocular samples may be a better way to obtain a rapid diagnosis in

patients suffering from unknown intraocular infectious disorders. As early treatments are also essential for infectious endophthalmitis, this method may help to ensure that patients receive timely and optimal treatments. However, this is currently a limited research tool and not widely available for clinical labs at the present time. As a next step, we will need to work on making these tests widely available to clinical labs as oppose to only having them in research labs. In the near future, it is assumed that a comprehensive PCR system for examining fungi, bacteria, parasites, and viruses will become available, and be able to be used in the diagnosis of ocular infectious disorders.

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ORIGINAL ARTICLE

Correlation between multiple *RET* mutations and severity of Hirschsprung's disease

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Abstract
Purpose The enteric nervous system (ENS), comprising neurons and glial cells, organized as interconnected ganglia within the gut wall, controls peristalsis and the production of secretions. The RET receptor tyrosine kinase is expressed throughout enteric neurogenesis and is required for normal ENS development. Humans with mutations in the RET locus have Hirschsprung's disease (HSCR), and mice lacking RET exhibit total intestinal aganglionosis. Although a number of mutations with the potential for causing HSCR have been reported, their precise correlation with phenotype and symptom severity in HSCR is not clearly understood. Our study investigates the correlation between mutations in the RET locus and symptom severity in HSCR.
Methods We performed a comprehensive nucleotide analysis of the RET coding region in 18 HSCR patients and 87 controls, performed cellular biological analysis by Western blotting using the expression vector, and analyzed cell proliferation with anti-Ki67 antibody under immunofluorescence confocal microscopy (ICM).
Results We identified three novel mutations, D489N, L769L, and V778D in the RET coding region in our HSCR patients. In the allelic distribution of D489N and L769L, the difference between HSCR patients and controls reached statistical significance ($p = 0.0373$ and $p = 0.0004$, respectively), whereas no statistical difference was observed in the allelic distribution of V778D ($p = 0.1073$). One HSCR patient who died from total colonic aganglionosis had a combination of homozygous mutation of D489N, L769L, and heterozygous mutation of V778D. Western blotting of full mutant RET from this patient showed significantly increased 150kD-band, which corresponds to the immature form compared with wild-type and single mutant RET. ICM showed that overexpression of full mutant RET significantly reduced cellular proliferation in comparison with wild-type and single mutant RET.
Conclusion A combination of mutations in the RET locus may correlate with symptom severity in HSCR as a consequence of reduced cellular proliferation secondary to altered maturation of RET.

Keywords Comprehensive exon nucleotide analysis · Maturation · Proliferation · RET tyrosine-type kinase · Hirschsprung's disease

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Introduction

Hirschsprung's disease (HSCR) is a congenital disorder of the enteric nervous system (ENS) and is characterized by the absence of intestinal ganglion cells in myenteric and submucosal plexuses. The length of the aganglionotic segment has been used to classify HSCR into short-segment type, rectosigmoid-type, long-segment type, extensive-type, and total-colon aganglionosis. Its incidence is

approximately 1/5,000 human live births, and has a male preponderance of 4:1 [1–3]. There is a spectrum of symptoms, the severity of which can vary widely.

In previous studies, several genes have been reported to be responsible for HSCR in humans; the RET gene [4], the GFRα1 gene [5], the EDNRB gene [6], the EDN4 gene [6], and the SOX10 gene [7], with the RET proto-oncogene being considered the major candidate gene for causing HSCR [1].

The RET gene encodes the RET receptor tyrosine kinase which is expressed in neural crest-derived cells and the kidneys. RET protein is required for normal ENS development [8] and is broadly divided into two forms. One is a 170 kDa mature glycosylated form, which is localized on cell membranes, and the other is a 150 kDa immature glycosylated form, which is localized in the cytoplasm and endoplasmic reticulum [9]. GFRα is necessary for activation of RET protein in the presence of their ligands such as glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin, and persephin [10]. Activating RET protein triggers the proliferation, differentiation, and survival of ENS cells [11, 12]. When there are mutations in the RET locus, there is abnormal development of the ENS, and mice lacking *Ret* fail to develop ENS and exhibit total intestinal aganglionosis [13, 14]. It has been previously reported that there is deterioration in the maturation of RET protein and its translocation to plasma membranes when there is mutation of the RET extracellular domain [15]. These results suggest that a decrease in matured RET protein on plasma membranes could be a potential cause of HSCR.

Although many studies have provided genetic and molecular evidence for the potential role of RET in HSCR, the precise correlation between molecular phenotype and symptom severity is not clearly understood. To investigate whether a combination of mutations in the candidate locus may contribute to symptom severity in HSCR, we firstly performed a comprehensive nucleotide analysis of common and rare variants of RET obtained from Japanese HSCR patients and constructed an expression vector containing multiple RET mutations identified from a patient with HSCR who died from total colonic aganglionosis, and then performed cellular biological analysis using this expression vector.

Materials and methods

Patients

In this study, 18 Japanese HSCR patients from unrelated families who were treated surgically at Juntendo University Hospital in Japan between 1997 and 2009 were the subjects for this study. A definitive diagnosis of aganglionosis was obtained from medical records. The control group comprised 87 students from Tokyo Medical and Dental University in Japan. They were unselected, unrelated, age- and sex-mismatched individuals. Informed consent was obtained from all participants. Methodology for collecting medical data and gene analysis were approved by the Research Ethics Committee of the Faculty of Medicine, Juntendo University (approval number 454).

DNA sequencing and mutation analysis

A comprehensive nucleotide analysis of the RET coding region from 18 HSCR patients and 87 controls was performed. Mutation analysis was performed by direct sequencing using an ABI Prism®3100 Genetic Analyzer (Life Technologies Japan, Tokyo, Japan). The specific primers used in this study are listed (Table 1).

Cloning

The full-length RET9-D489N, RET9-L769L, RET9-V778D, and RET9-D489N/L769L/V778D were constructed using wild-type human RET9 as a template, using KOD with a mutagenesis system (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Wild-type human RET9 and mutant type RET9 were inserted into pcDNA3 vector (Life Technologies Japan, Tokyo, Japan) tagged HA. All expression vectors were confirmed by DNA sequencing. The primers used for mutagenesis are listed (Table 2).

Cell culture and transfection

COS7 (African green monkey kidney fibroblast) cells were cultured in Dulbecco's-modified Eagle's medium (DMEM) (Life Technologies Japan, Tokyo, Japan) containing 10 % fetal bovine serum (FBS) (PAA Laboratories, Pasching, Austria), supplemented with 100 U/mL penicillin–streptomycin (Life Technologies Japan, Tokyo, Japan). For RET expression analysis by Western blotting, COS7 cells were plated to a 60 mm diameter dish at a density of 5×10^5 cells per well and transfected with the expression vector using Lipofectamine2000 (Life Technologies Japan, Tokyo, Japan) for 24 h. For proliferation assay by immunostaining, COS7 cells were plated onto the cover glass of a 24 well-plate at a density of 5×10^4 cells per well and transfected in the same way.

Western blotting

Cells were lysed in sodium dodecyl sulphate (SDS) sample buffer by sonication. The lysates were subjected to SDS-polyacrylamide gel electrophoresis, and transferred to