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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 (\pm SD) was 60 (\pm 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 Ampliqa 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'-ttagcttagatgRaRtttaccacc-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. flavus* (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 fungemia (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 fungemia (cases 30, 77, 161, and 355) initiated antimycotic drug therapy after receiving positive PCR results (Table 2).

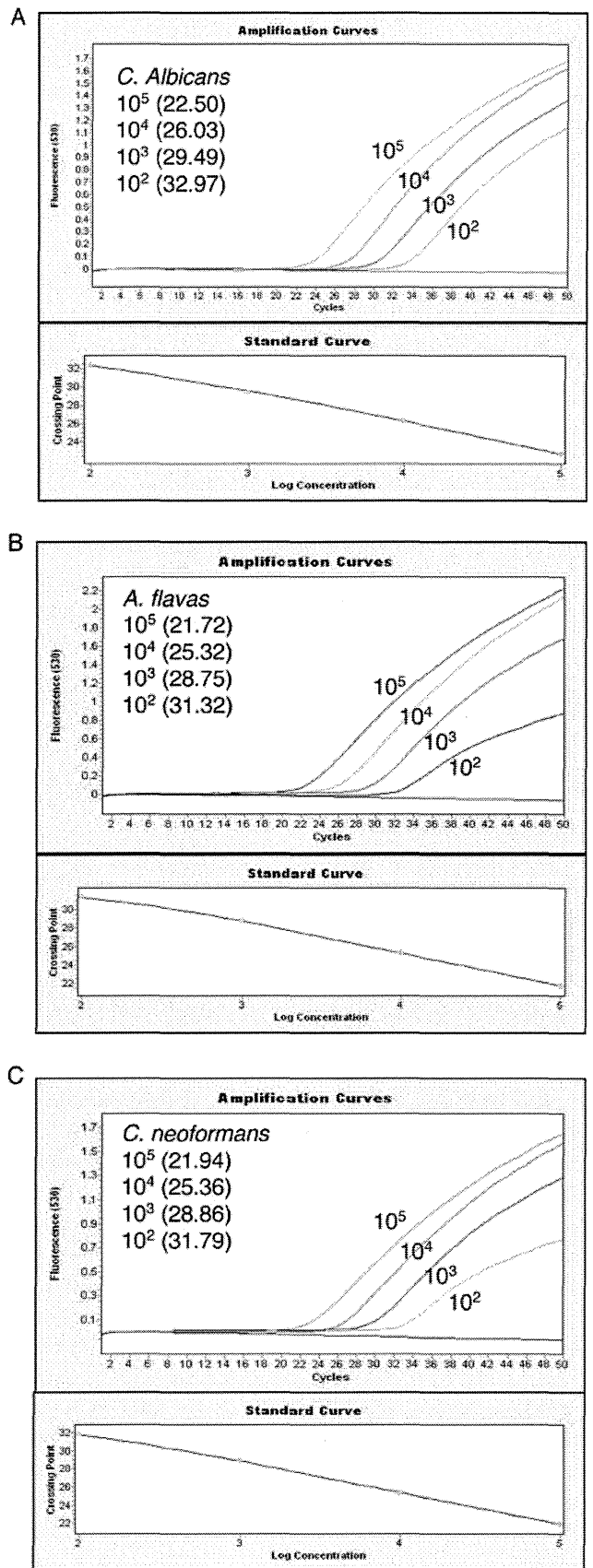
The diagnostic parameters of sensitivity, specificity, positive predictive value, and negative predictive value of the PCR examinations for the diagnosis of fungal endophthalmitis were calculated to be 0.833, 0.998, 0.909, and 0.996, respectively.

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. flavus*, **b**), and *Cryptococcus* (*C. neoformans*, **c**) species. The number in the parenthesis indicates the cycle threshold (Ct) value in quantitative PCR



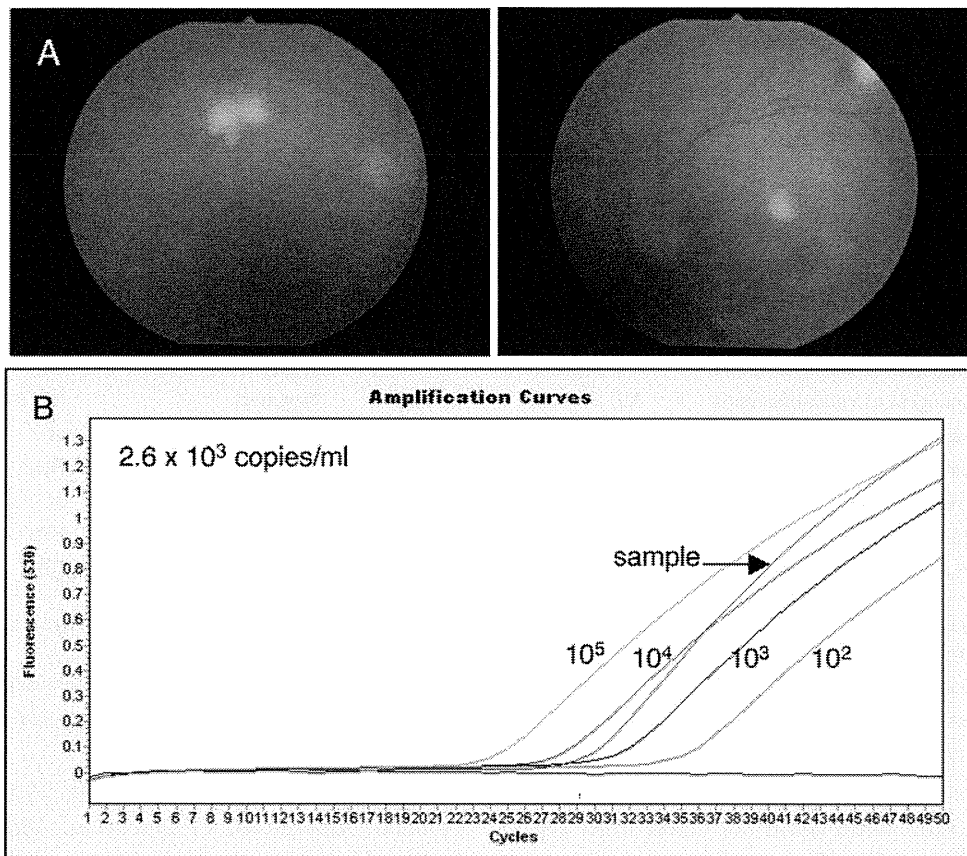


Fig. 2 A representative PCR result from a patient with endogenous endophthalmitis related to *Aspergillus* infection (case 179 in Table 1). A 74-year-old woman with type II diabetes mellitus was treated for splenic cancer. After surgery, she used intravascular catheters. She reported blurred vision and 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	<i>Candida albicans</i>	BDG - negative
77	85/M	Endogenous endophthalmitis	VF	41.3	7.6 × 10 ³	<i>Candida</i> spp.	nt
161	75/M	Endogenous endophthalmitis	VF	57.8	2.8 × 10 ⁵	<i>Candida albicans</i>	nt
179	74/F	Endogenous endophthalmitis	AH	29.1	2.6 × 10 ³	Negative	<i>Aspergillus</i> 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	<i>Cryptococcus neoformans</i>	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	<i>Candida</i> 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; <i>C. acbicans</i>
490	49/M	Endogenous endophthalmitis	AH	43	6.6 × 10 ⁴	<i>Candida albicans</i>	BDG - 449; <i>C. acbicans</i>

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

^aFungal 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*, *Microsporium*, *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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Virological Analysis in Patients with Human Herpes Virus 6–Associated Ocular Inflammatory Disorders

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PURPOSE. To determine whether human herpes virus 6 (HHV-6) genomic DNA and mRNA can be detected in ocular samples from patients with inflammatory disorders, and whether viral replication is involved in the development of inflammation in the eye.

METHODS. After informed consent was obtained, ocular fluid samples (aqueous humor and vitreous fluids) were collected from 350 patients with uveitis or endophthalmitis. Corneal samples were also collected from 65 patients with corneal infections. Multiplex PCR was performed to screen ocular samples from the patients for HHV-1 to HHV-8. Samples were also assayed for HHV-6 DNA using quantitative real-time PCR. Primers for nested RT-PCR were designed to detect amplification of mRNA (HHV-6 A IE1 U90).

RESULTS. PCR results indicated a total of seven patients with uveitis or endophthalmitis (7/350, 2%+) and a single patient with corneal inflammatory disease were positive for HHV-6 DNA (1/65, 1.5%+). These eight patients had high copy numbers of HHV-6 DNA, with values ranging from 4.0×10^3 to 5.1×10^6 copies/mL. Real-time PCR analysis indicated that two of these cases were HHV-6 variant A and six cases were variant B. In addition, HHV-6 mRNA was clearly detected in vitreous cells collected from one of the patients, suggesting that viral replication may occur in the eye.

CONCLUSIONS. Our results indicate that HHV-6 infection/reactivation is implicated in ocular inflammatory diseases. (www.umin.ac.jp/ctr/index/htm number, R000002708.) (*Invest Ophthalmol Vis Sci.* 2012;53:4692–4698) DOI:10.1167/iovs.12-10095

Human herpesvirus 6 (HHV-6) is the causative agent of Hexanthea subitum in children and has been associated with a number of inflammatory and neurological disorders

worldwide. It has been implicated in hepatitis, pneumonitis, and severe infections of the central nervous system in both immunosuppressed and immunocompetent patients. HHV-6 can reactivate from its latent form after primary infection. In the case of eye diseases, it has been implicated in AIDS-associated retinitis,^{1–3} uveitis,^{4–8} corneal inflammation,⁹ and optic neuropathy.^{10–12} Two variants of HHV-6 have been identified. HHV-6A is less often associated with disease and has a greater predilection for neural cells than HHV-6B.¹³ Although HHV-6A DNA is frequently found in the nervous system of infected adults, HHV-6B DNA is rarely present in ocular fluids, although it is found in most documented primary HHV-6 infections.

Diagnosis of clinically relevant HHV-6 can be challenging due to the high prevalence of infection and viral persistence. Detection of viral nucleic acids may indicate active or latent infections, depending on the clinical setting and specimens tested. Quantitative PCR methods have been established to detect active infections. Detection of HHV-6 DNA in plasma or serum is indicative of active replication and is therefore more directly interpretable.^{14,15} Using these PCR techniques, several investigators previously reported that HHV-6 genomic DNA is found in ocular inflammatory diseases, including infectious uveitis and endophthalmitis^{1–8}; however, involvement of HHV-6 in ocular infections has not yet been clearly demonstrated.

Therefore, we designed experiments to investigate whether ocular samples from patients with various ocular inflammatory disorders contain HHV-6 genomic DNA, whether ocular samples from noninflammatory patients also contain HHV-6 DNA, whether positive cases are either HHV-6 variant A or B, and whether HHV-6 mRNA as well as a high copy numbers of HHV-6 DNA can be detected in positive samples.

MATERIALS AND METHODS

Subjects

The first patient group was examined between 2006 and 2010 at the Tokyo Medical and Dental University Hospital, Kyoto Prefectural University Hospital, and Shinkawabashi Hospital in Japan. After informed consent was obtained, ocular fluid samples were collected from patients with uveitis (infectious and noninfectious) or endophthalmitis. This group included consecutive patients with uveitis or endophthalmitis ($n = 350$), including a previously HHV-6-positive severe panuveitis case.⁷ Corneal tissues were also collected from patients with ocular surface diseases (e.g., keratitis, $n = 65$). At this time, we excluded ocular tumor diseases (e.g., intraocular lymphoma) from the patient group.

In addition to the patient group, we also analyzed samples from a control group. A total of 100 samples (50 aqueous humor and 50 vitreous fluids) were collected from patients who did not have any type of ocular inflammation (age-related cataract, macular edema, retinal

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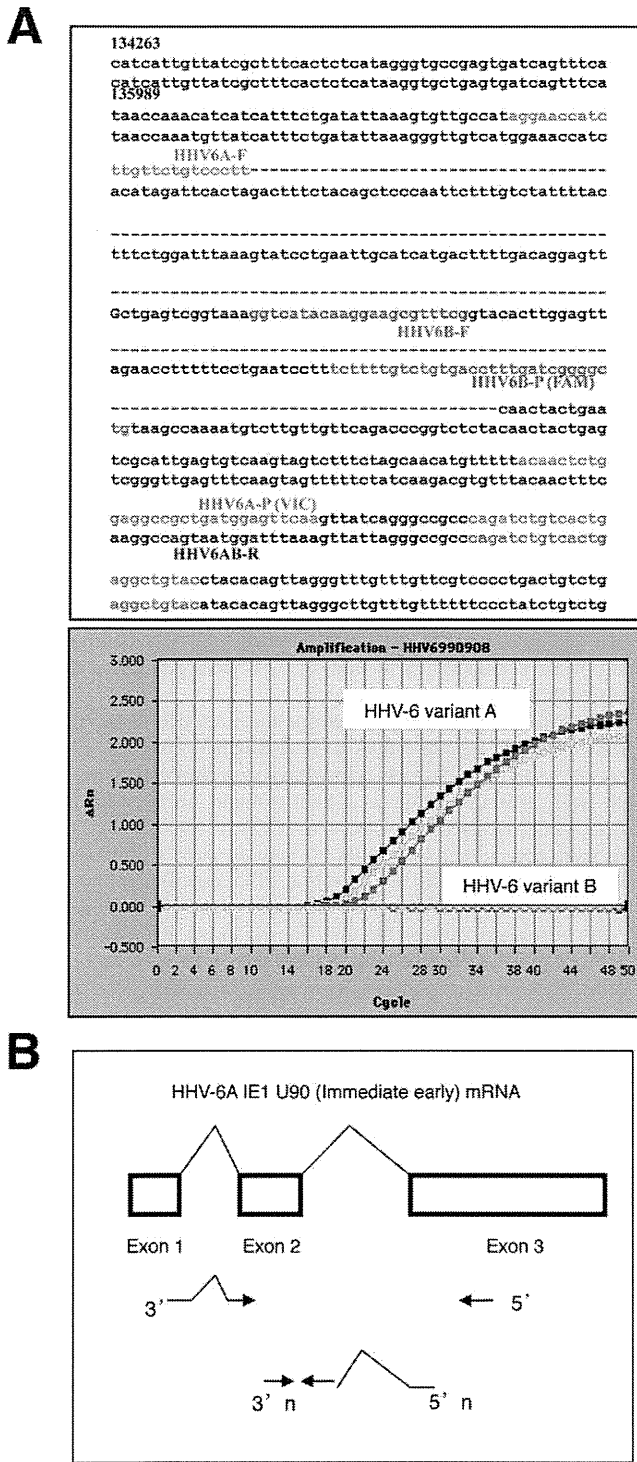


FIGURE 1. Amplification of HHV-6-specific DNA and mRNA. (A) TaqMan probes and primers used to amplify HHV-6 DNA (HHV-6A and HHV-6B). HHV-6 subtypes were identified using PCR with variant-specific primers and probes (lower graph). (B) Nested RT-PCR primers were designed to amplify HHV-6A mRNA.

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. A clinical registration was conducted and information is available at www.umin.

TABLE 1. Clinical Findings in Patients with HHV-6-Associated Ocular Inflammatory Disorders

Case	Age / Sex	Eye	Initial Diagnosis	VA	IOP	Cornea	AC	KPs	VO	Fundus	Bacterial Examination*	Final Diagnosis
1	75 / Male	R	Pan-uveitis	0.02	15	None	Hypopyon	Mutton fat	Grade III	Retinal exudates	Culture (-) / PCR (-)	Ocular toxocariasis
2	64 / Female	L	Corneal endotheliitis	0.5	33	Edema	Cell 2+	Mutton fat	None	None	PCR (-)	HSV-1 corneal endotheliitis
3	70 / Male	L	Bacterial endophthalmitis	sl-	35	None	Hypopyon	Fine	Grade III	Retinal necrosis	Culture (+) / PCR (+)	Endogenous endophthalmitis
4	74 / Female	R	Idiopathic uveitis	0.8	16	None	Cell 1+	None	Grade II	None	PCR (+)	Late postoperative endophthalmitis
5	79 / Female	L	Bacterial endophthalmitis	mm	19	None	Hypopyon	Fine	Grade II	Retinal exudates, hemorrhage	Culture (+) / PCR (+)	Acute postoperative endophthalmitis
6	71 / Female	L	Necrotic retinitis	0.04	12	None	None	None	None	Retinal necrosis, hemorrhage	PCR (-)	Cytomegalovirus retinitis
7	24 / Female	L	Posner-Schlossman synd.	1.2	24	None	Cell 1+	Mutton fat	None	None	PCR (-)	Idiopathic uveitis
8	22 / Male	R	Keratitis	0.7	15	Infiltration	Cell 1-	None	None	None	Culture (-) / PCR (+)	Bacterial keratitis

* Bacterial examination: Results for bacterial culture and/or PCR (bacterial 16S rDNA). AC, anterior chamber; KPs, keratic precipitates; VA, visual acuity by Landolt Chart; VO, vitreous opacity.

TABLE 2. Virological Analysis and Treatment in Patients with HHV-6-Associated Ocular Inflammatory Disorders

Case	Ocular Sample	HHV Genome	Viral Copy No. by Real-Time PCR	HHV-6A or B	Treatment
1	Aqh	HHV-6	HHV-6: 2.4×10^6 copies/mL	HHV-6A	PSL, PPV, VCV, VGV
	VF	HHV-6, EBV	HHV-6: 2.0×10^4 copies/mL, EBV: <50 copies/mL		
2	Aqh	HHV-6, HSV-1	HHV-6: 7.5×10^3 copies/mL, HSV-1: 2.8×10^5 copies/mL	HHV-6B	VGV
3	VF	HHV-6	HHV-6: 5.1×10^6 copies/mL	HHV-6B	PPV, SA, IAI
4	VF	HHV-6	HHV-6: 1.1×10^4 copies/mL	HHV-6B	PPV, VGV
5	VF	HHV-6	HHV-6: 1.1×10^6 copies/mL	HHV-6B	PPV, SA, Betametasone
6	VF	HHV-6, CMV	HHV-6: 4.4×10^4 copies/mL, CMV: 1.6×10^6 copies/mL	HHV-6A	VGV
7	Aqh	HHV-6	HHV-6: 4.0×10^3 copies/mL	HHV-6B	None
8	Cornea	HHV-6	HHV-6: 3.9×10^6 copies/ μ g · DNA	HHV-6B	Antibiotics

Aqh, aqueous humor; IAI, intravitreal antibiotic injection; PPV, pars plana vitrectomy; PSL, prednisolone; SA, systemic antibiotics; VCV, valacyclovir; VF, vitreous fluids; VGV, valganciclovir.

ac.jp/ctr/index/htm with study number of R000002708. The study started in April 2006 and terminated in April 2010.

PCR

DNA was extracted from samples using an E21 virus minikit (Qiagen, Valencia, CA) installed on a robotic workstation for automated purification of nucleic acids (BioRobot E21, Qiagen). HHV genomic DNA in ocular samples was detected using two independent PCR assays: a qualitative multiplex PCR and a quantitative real-time PCR.¹⁶

The multiplex PCR was designed to qualitatively measure genomic DNA of eight human herpes viruses as follows: herpes simplex virus type 1 (HSV-1), type 2 (HSV-2), Varicella-zoster virus (VZV), Epstein-Barr virus (EBV), cytomegalovirus (CMV), and human herpes virus 6 (HHV-6), 7 (HHV-7), and 8 (HHV-8). PCR was performed using a LightCycler (Roche, Rotkreuz, Switzerland). Primers for HHV-6 were as follows: Forward - ACCCGAGAGATGATTTTGCG and Reverse - GCAGAAGACAGCAGCGAGAT. Probes were used as follows: 3'^{FITC}-TAAGTAACCGTTTTTCGTCCTCA and LcRed705-5'-GGGTCAATTTATGTTATAGA. These primers and probes do not distinguish between HHV-6A and B. PCR conditions, primers, and probes specific for other HHV have been described previously.¹⁷

Real-time PCR was performed for detection of HHV only, following identification of genomic DNA by multiplex PCR. Real-time PCR was performed using Ampliqaq Gold and the Real-Time PCR 7300 system (ABI, Foster City, CA). The sequence of the HHV-6 primers and probes are as follows: Forward - GACAATCACATGCCTGGATAATG and Reverse - TGTAAGCGTGTGGTAATGTACTAA. The probe was AGCAGCTGGCGAAAAGTGCTGTGC. The primers and probes of other herpes viruses and the PCR conditions have been described previously.^{16,17} These primers and probes do not distinguish between HHV-6A and B. TaqMan probes and primers used in the HHV-6 DNA amplifications, HHV-6 type A and HHV-6 type B, are shown in Figure 1A. The value of viral copy number in the sample was considered to be significant when more than 50 copies/mL were observed.

RT-PCR

The primers for nested RT-PCR were designed to detect mRNA (HHV-6 A IE1 U90 immediate early) as follows: first PCR Forward - GATGAACGTATGCAAGACTACC and ATGAACATGGATTGTTGCTG and Reverse - CAGCGACTGAGCAGCTA; nested PCR Forward - CCGATCCAATGATGGAAGAA and Reverse - CAGCGACTGAGCAGCTA (Fig. 1B). A one-step RT-PCR was performed on 100 ng of total RNA with 0.5 μ M of each primer and SuperScript III One-Step RT-PCR with platinum Taq (Life Technologies Co., Tokyo, Japan) in a final volume of 50 μ L. Samples were reverse transcribed for 30 minutes at 54°C and amplified for 40 cycles consisting of denaturation for 15 seconds at 94°C, annealing for 30 seconds at 54°C, and polymerization for 20 seconds at 72°C. Following identification of a PCR product of 340 bp, nested PCR was performed on 1 μ L of the first PCR solution using 0.5

μ M of each primer and 200 mM deoxynucleotide triphosphates and 1.25 U of Taq DNA polymerase (Thermo Fisher Scientific, Tokyo, Japan). Monoclonal antibody (anti-taq high: Toyobo Life Science, Tokyo, Japan) was used at 0.25 μ g in a buffer containing 75 mM Tris-HCl (pH = 8.8), 0.01% Tween-20, 20 mM (NH₄)₂SO₄, and 1.5 mM MgCl₂ in a final volume of 50 μ L. Twenty cycles of amplification consisting of denaturation for 15 seconds at 94°C, annealing for 30 seconds at 55°C, and polymerization for 15 seconds at 72°C were performed to give a positive PCR product of 198 bp.

All ocular samples were tested for the presence of β -actin as an internal control. β -Actin mRNA RT-PCR was performed on 100 ng of total RNA with 0.5 μ M each primer and SuperScript III One-Step RT-PCR with platinum Taq in a final volume of 50 μ L (Forward-CTTCCTTCTGGGCAT and Reverse-TCTTCATTGTGCTGGGT). Samples were reverse transcribed for 30 minutes at 55°C followed by 40 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and polymerization for 1 minute at 72°C on a thermal cycler TP-400 instrument (Takara Bio Inc., Tokyo, Japan). Raji cell lines were used as a positive control, and MOLT-4 cells were used as a negative control. PCR products were analyzed using 2% agarose gel electrophoresis and ethidium bromide staining and the positive product was 215 bp.

RESULTS

Detection of HHV-6 Genomic DNA in Patients with Uveitis, Endophthalmitis, and Ocular Surface Diseases

We first performed multiplex PCR to screen for 8 HHVs after collecting intraocular samples from patients with various ocular inflammatory diseases. PCR results indicated that 7 (2%) of 350 patients with uveitis or endophthalmitis were positive for HHV-6 DNA. In addition, 1 (1.5%) of 65 patients tested positive for HHV-6 in a corneal tissue sample. These HHV-6-positive cases together with clinical findings are summarized in Tables 1 and 2. These eight HHV-6-positive patients were clinically suspected to have HHV-6-associated infectious diseases based on the detection of HHV-6 genome in ocular fluid or corneal tissue samples. HHV-6 DNA was not detected in any of the 100 control samples that were collected from patients without ocular inflammation.

The clinical features observed in HHV-6-positive cases at their initial presentation are summarized in Table 1. Almost all of the patients with uveitis and endophthalmitis had active ocular inflammation, that is, there were anterior chamber cells (except case 6), keratic precipitates (except cases 4 and 6), vitreous opacity (except cases 2 and 7), and fresh retinal exudates/necrosis (except cases 2, 4, and 7). In the single patient with HHV-6⁺ keratitis (case 8 in Table 1), corneal

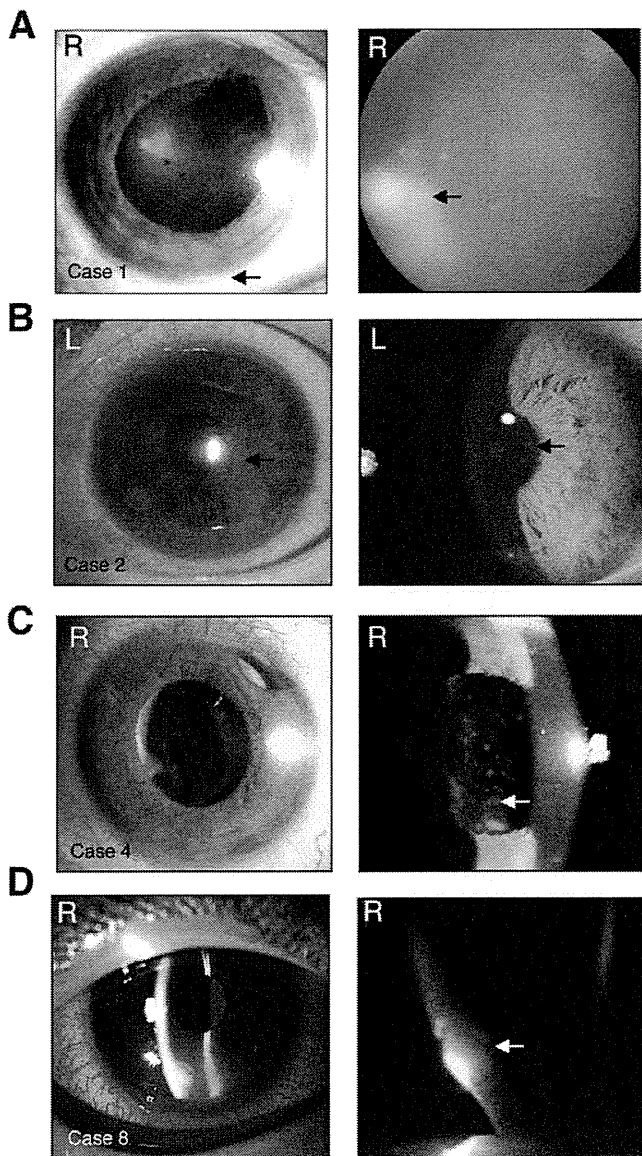


FIGURE 2. Slit-lamp and fundus photographs for HHV-6 infections. (A) Case 1: A case of ocular toxocariasis. Slit-lamp examination of right eye (RE) disclosed ciliary injection, moderate mutton-fat keratic precipitates (KPs), and severe anterior chamber cells with hypopyon (*arrow*). Funduscopic examination of the RE revealed dense vitreous opacities and yellowish white massive retinal lesions (*arrow*) in the peripheral fundus. HHV-6 DNA was detected in both aqueous humor and vitreous samples. (B) Case 2: A case of HSV-1-associated corneal endotheliitis. Slit-lamp examination of left eye (LE) disclosed pigmented mutton-fat-like KPs with high intraocular pressure, mild anterior chamber cells, and small-size corneal stromal edema (*arrow*). HSV-1 and HHV-6 DNA were detected in aqueous humor, but other HHV-DNA, such as VZV and CMV, was not detected. (C) Case 4: A case of late postoperative endophthalmitis. This patient with Vogt-Koyanagi-Harada disease had postcataract surgery 6 months earlier. Slit-lamp examination of RE disclosed ciliary injection and mild anterior chamber cells. White plaque (*arrow*) on the intraocular lens and mild inflammation were seen, and an aqueous humor sample was obtained. HHV-6 DNA and *Propionibacterium acnes* DNA were detected in the aqueous humor sample. The final diagnosis was *P. acnes*-associated late postoperative endophthalmitis. (D) Case 8: A case of bacterial keratitis. Slit-lamp examination of RE disclosed keratitis (*arrow*) with ciliary injection. A corneal infiltration with epithelial defect was observed and a high copy number of HHV-6 DNA was detected in corneal tissue samples.

infection, such as corneal epithelial ulcer and ciliary injection, was indicated. Representative findings including slit-lamp or fundus photographs for HHV-6-positive cases are shown in Figure 2. In addition, ocular samples from all patients were subjected to bacterial examinations, including conventional bacterial culture and bacterial broad-range PCR (bacterial 16S rDNA)¹⁸ (Table 1). The final diagnoses were as follows: case 1, ocular toxocariasis; case 2, HSV-1 corneal endotheliitis; case 3, endogenous endophthalmitis; case 4, late postoperative endophthalmitis; case 5, acute postoperative endophthalmitis; case 6, CMV retinitis; case 7, idiopathic uveitis; case 8, bacterial keratitis (Table 1).

We next summarized the virological analysis of ocular samples from these eight HHV-6-positive patients (3 aqueous humor, 5 vitreous fluids, and 1 corneal tissue) in Table 2. Multiplex PCR was used to detect HHV infection (HSV-1, HSV-2, VZV, EBV, CMV, HHV-6, HHV-7, and HHV-8). HHV-6 was found together with EBV (only case 1), HSV-1 (only case 2), or CMV (only case 6). Figure 3 is representative of the results of the multiplex PCR where HHV-6 DNA was detected in aqueous and vitreous fluid from case 1. HHV DNA in nine ocular samples from eight cases was also measured by real-time PCR. These patients had high copy numbers of HHV-6 DNA, with values ranging from 4.0×10^3 to 5.1×10^6 copies/mL (Table 2), suggesting that viral replication may occur in the eye. Following diagnosis, 4 patients received antiviral treatment (i.e., valacyclovir or valganciclovir), which controlled their ocular inflammation (Table 2).

Detection of HHV-6 Variant A or B in Patients with HHV-6-Associated Ocular Inflammatory Disorders

HHV-6 can be classified into two groups: a variant A (HHV-6A) and a variant B (HHV-6B).¹⁵ Distinguishing between HHV-6 subtypes is mainly accomplished using PCR techniques, including melting curve¹⁹ or variant-specific primers.²⁰ Therefore, we next determined whether the HHV-6-positive cases were HHV-6A or B using real-time PCR. In this study, we designed a probe and primers for use in the HHV-6 DNA amplification. The paired primers and TaqMan probes used for detection of HHV-6A and HHV-6B are shown in Figure 1A. By using several different primers and probes, we were able to detect each of these HHV-6 types separately (Fig. 1A). The PCR results from case 1 showed that intraocular samples included HHV-6A but not HHV-6B DNA (Fig. 4). Final analysis with quantitative PCR indicated that two of the cases were positive for HHV-6A and six cases were positive for HHV-6B (Table 2).

Detection of HHV-6 mRNA in Intraocular Samples

RT-PCR has previously been used on mRNA from peripheral blood mononuclear cells to detect actively replicating virus.²¹ We therefore tested ocular samples for the presence of HHV-6 mRNA. Various samples, such as aqueous humor, vitreous fluid, retinal membrane tissues, and collected vitreous cells from an HHV-6A-positive case (case 1), were available for the RT-PCR assay. We designed primers to amplify mRNA using a nested RT-PCR (HHV-6 A IE1 U90, Fig. 1B). As revealed in Figure 5, HHV-6A mRNA was clearly detected in vitreous cell samples, but other ocular samples from the same patient were all negative.

DISCUSSION

In this study, we demonstrate that seven patients with uveitis or endophthalmitis were positive for HHV-6 DNA. In addition, one patient with infectious keratitis was also found to be HHV-6-positive. These patients had high copy numbers of HHV-6

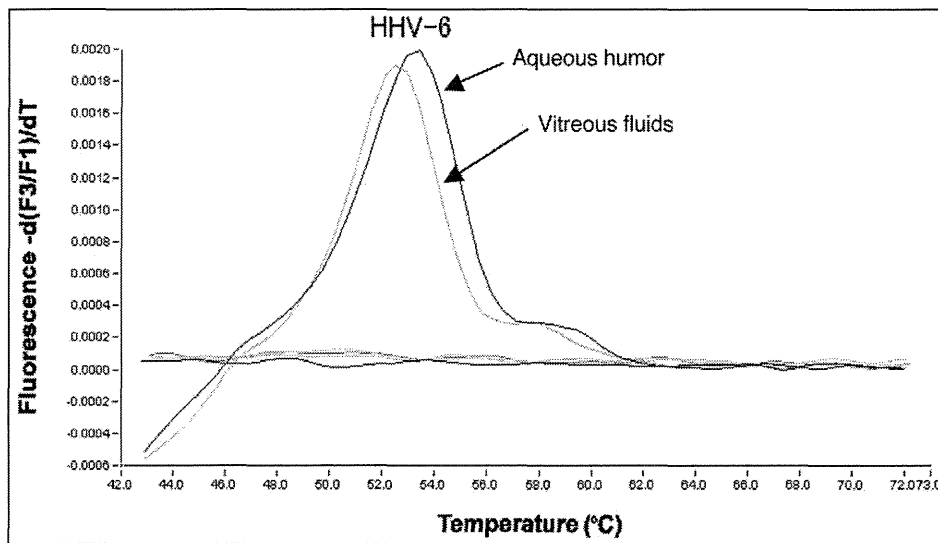


FIGURE 3. Results for multiplex PCR in a patient with HHV-6-positive uveitis. A significant positive curve was seen at 52°C, indicating detection of HHV-6 genomic DNA in the ocular fluids (case 1). DNA from other herpes viruses, such as HSV1, HSV2, VZV, EBV, CMV, HHV7, and HHV8, was not detected in this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

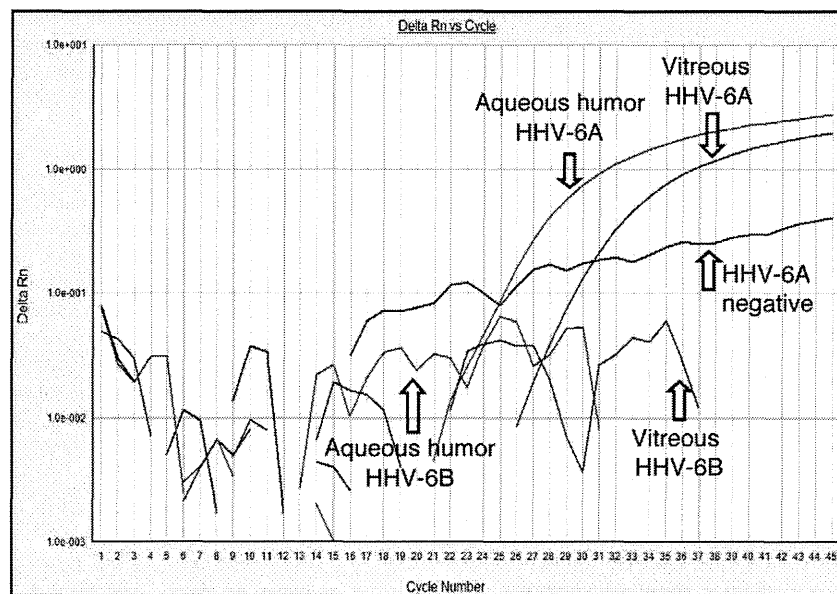


FIGURE 4. Detection of HHV-6 DNA by quantitative real-time PCR. The real-time PCR results for the samples from case 1 showed that intraocular samples, such as aqueous humor and vitreous fluids, contained a high copy number of HHV-6A DNA, but not HHV-6B DNA.

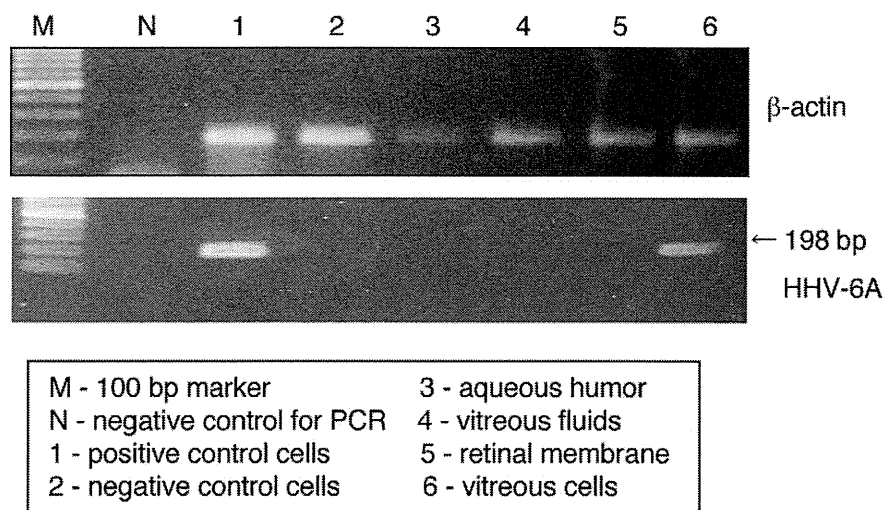


FIGURE 5. Detection of HHV-6 mRNA in intraocular samples. HHV-6A mRNA was detected in samples from vitreous cells, but other ocular samples, such as aqueous humor, vitreous fluids, and retinal membrane tissues were all negative (*lower image*). All samples, including control RNA, were positive for β -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 HHV-6-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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Examination of the 16S-23S rRNA Intergenic Spacer Sequences of '*Candidatus Mycoplasma haemobos*' and *Mycoplasma haemofelis*

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ABSTRACT. The intergenic spacer region between the 16S and 23S rRNA genes of mycoplasmas has been used for a genetic marker for identification of the species. Here we show the intergenic spacer regions of two hemotropic mycoplasmas, *Mycoplasma haemofelis* and '*Candidatus Mycoplasma haemobos* (synonym: '*C. M. haemobovis*')' are also useful for classification of this particular group of mycoplasmas. The spacer region of *M. haemofelis* and '*C. M. haemobos*' consisted of 209 and 210 base pairs, respectively, and both lacked the spacer tRNA genes. Phylogenetic analysis suggested a monophyletic relationship among hemoplasmas and *M. fastidiosum*. A hypothetical secondary structure predicted in the spacer regions tentatively assigned the boxA and boxB motifs peculiar to the members of the genus *Mycoplasma*. *M. haemofelis* and '*C. M. haemobos*' possessed a stem-loop structure in common, despite the presence of a palindromic nucleotide substitution in the stem region.

KEY WORDS: 16S-23S rRNA, haemoplasma, mycoplasma.

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Hemoplasmas, hemotropic mycoplasmas, are causative of infectious anemia in animals, but have never been cultured *in vitro* [16, 17]. The members of the genus *Mycoplasma* have only one or two operons for the rRNA gene [1]. The genes encoding for rRNA molecules of the genus *Mycoplasma* are usually organized in the order of 5'-16S-23S-5S-3', in which the individual rRNA genes are separated by internal transcribed spacer (ITS) regions [22], except for *Mycoplasma suis* [14, 19]. The ITS region between the 16S and 23S rRNA genes of the genus *Mycoplasma* has been shown to lack spacer tRNA genes so far [15] and to be variable in sequence and length depending on the species [27]. Thus the ITS region has been used as a genetic marker for comparing phylogenetic relationships of genetically closely related species among not only the mycoplasmas [5], but also other bacterial species [3, 12, 13, 25]. Therefore, analyses of ITS regions may provide useful information for defining the genealogical positions of hemoplasmas. In the present study, we examined the ITS sequences of two hemoplasma species, *Mycoplasma haemofelis* [16, 17] and '*Candidatus Mycoplasma haemobos* (synonym: '*C. M. haemobovis*')' [24], both causative of infectious anemia in cats and cattle, respectively.

Heparin or EDTA-anticoagulated blood samples collected from cats in Morioka and cattle in Miyazaki, Japan, were kept at -20°C prior to analysis. Total DNA was extracted from 200 µl of the anticoagulated whole blood

samples by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, eluting into 200 µl of buffer AE, and stored at -20°C until examination in the PCR assay. Two hemoplasma species, *M. haemofelis* and '*C. M. haemobos*', identified in the present study according to the methods as described previously [2, 18], were further subjected to analysis of the ITS region between the 16S and 23S rRNA genes. No mixed infection was apparent in each animal that these hemoplasmas were detected.

ITS region was amplified from the total DNA extracted from '*C. M. haemobos*' strain Gandai1 and *M. haemofelis* strain Gandai2. PCR amplification of the isolated DNA was carried out at 94°C for 30 sec, 55°C for 2 min, and 72°C for 2 min for 30 cycles using forward (5'-GTTCCAGGTCT-TGTACACA-3') and reverse (5'-CAGTACTTGTTCAC-TATCGGTA-3') primers as described previously [4]. The PCR products were fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts, Rockland, Me., U.S.A.) in TAE (40 mM Tris, pH8.0, 5 mM sodium acetate, 1 mM disodium ethylenediaminetetraacetate) buffer at 50 volts for 60 min. After electrophoresis, the gels were stained in ethidium bromide solution (0.4 µg/ml) for 15 min and visualized under UV transilluminator. ITS regions of only *M. haemofelis* and '*C. M. haemobos*' were successfully amplified by this PCR. DNA in a clearly visible band was extracted by using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, Calif., U.S.A.). The GenBank/EMBL/DBJ accession numbers for the 16S-23S rRNA intergenic spacer sequence of '*C. M. haemobos*' strain Gandai1 and *M. haemofelis* strain Gandai2 are AB638407 and AB638408,

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respectively. Reason for unsuccessful amplification of ITS of other hemoplasma species by this PCR remains unidentified. Mismatches in PCR primers were most likely in these cases.

The ITS region between the 16S and 23S rRNA genes of the genus *Mycoplasma* is well conserved within a species and used for a genetic marker for identification and classification of mycoplasmas [5]. ITS regions of *M. haemofelis* and '*C. M. haemobos*' were consisting of 209 and 210 base pairs, respectively. The nucleotide sequences of the ITS in these 2 species showed moderate similarity with that of *M. haemomuris* previously determined [8], and shared a consensus motifs called boxA and boxB in common (Fig. 1). The boxA, originally found at upstream regions of the *nut* site of the lambda phage genome, is considered to be a *cis*-acting element for the *Escherichia coli* NusA protein, a transcription control factor [20]. The conserved nature of the boxA sequence among these different species may implicate an important role in survival mechanisms, though the exact function of this motif in the ITS remains unknown [11]. The boxB sequence previously reported in mycoplasmas [5] was also assigned about ten nucleotides upstream of the boxA sequence of both the hemoplasma species. Presence of these particular motifs suggests that ITS might act as a controlling element for transcription of the rRNA operon in hemoplasmas. No spacer tRNA gene was identified within the ITS of *M. haemofelis* and '*C. M. haemobos*', and this is

a common feature in line with the other species of the genus *Mycoplasma* [27]. Transposase gene has been demonstrated in ITS of *M. imitans* [10], but such feature was not the case in these two hemoplasma species.

The nucleotide sequences of ITS regions of both the hemoplasmas were compared with authentic mycoplasma species in phylogenetic analysis. In the present study, we explored the genealogical relatedness among 20 mycoplasma species including not only ureaplasmas but also three hemoplasma species, *M. haemomuris*, *M. haemofelis*, and '*C. M. haemobos*'. The comprehensive phylogenetic tree suggested the 20 mycoplasma sequences were divided into three clusters consisting of Hominis, Pneumoniae and Fastidiosum groups (Fig. 2). Hemoplasmas formed a monophyletic clade in the Fastidiosum group in the present study, allowing more precise assignment of hemoplasmas as compared with phylogenetic analysis based on the 16S rRNA sequences [16, 24]. Although the Hominis and Pneumoniae groups in mycoplasmas have already been established in the phylogenetic analyses based on the 16S rRNA gene as well as on the RNase P RNA gene [21], we showed a monophyletic relationship among hemoplasmas and *M. fastidiosum* by phylogenetically analyzing ITS regions. Our findings support that given hemoplasmas are members of the class *Mollicutes*.

The secondary structures of the ITS were predicted according to the algorithm of Zuker and Stiegler [28]. Five

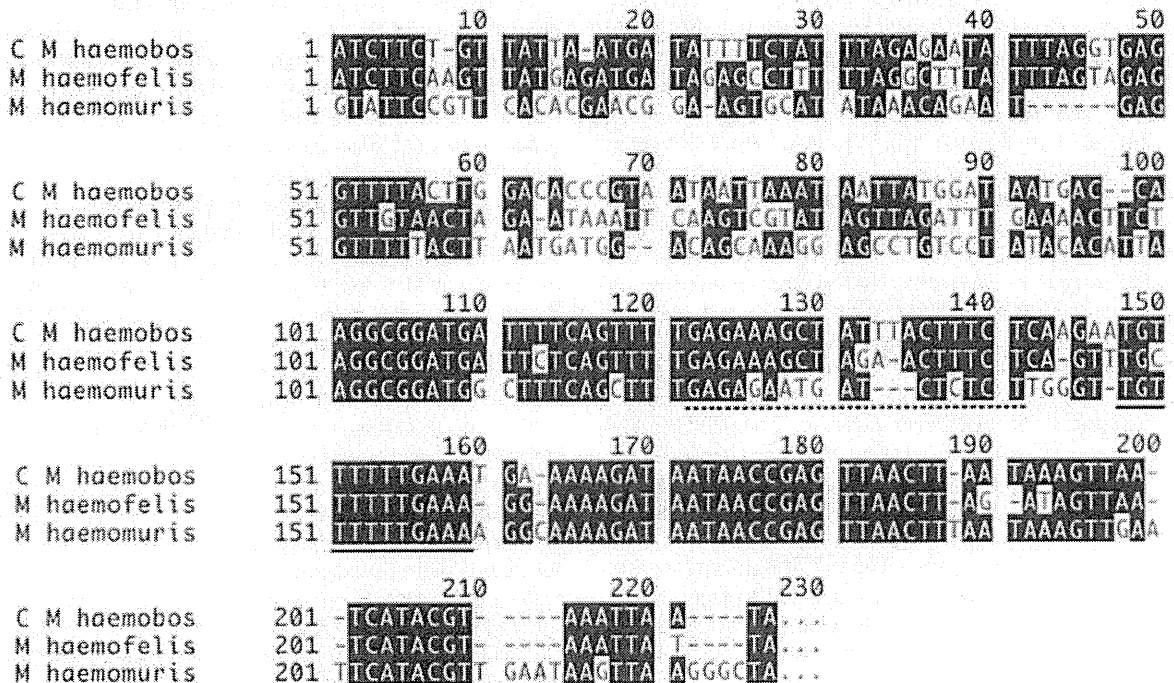


Fig. 1. Nucleotide sequence alignment of ITS from the three hemoplasma species, *M. haemofelis*, '*C. M. haemobos*' and *M. haemomuris*. The nucleotide sequence of *M. haemomuris* was obtained from the previous report [8]. Nucleotide sequence numbers are given from a consensus sequence. Homologous nucleotides are shown as inverted characters. Dashes indicate nucleotide gaps between adjacent nucleotides introduced for the alignment. BoxA is underlined, and boxB is shown by a dotted line.

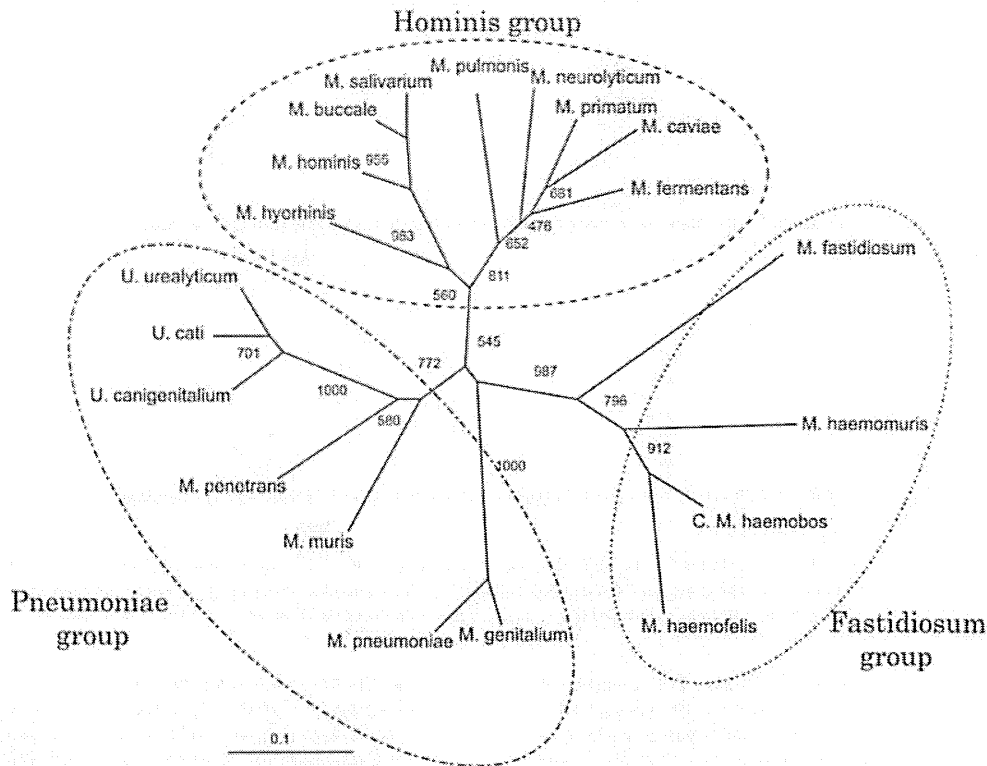


Fig. 2. Unrooted phylogenetic tree based on the ITS comparison, suggesting a monophyletic relationship among hemoplasmas and *M. fastidiosum*. Following nucleotide sequences were obtained from the DNA databases and accession number is given in parenthesis. They are *M. salivarium* PG20 (X58558), *M. pulmonis* m53 (X58554), *M. neurolyticum* Sabin Type A (X58552), *M. buccale* CH20247 (D89504), *M. primatum* HRC292 (D89509), *M. caviae* G122 (D89505), *M. hominis* PG21 (X58559), *M. fermentans* PG18 (X58553), *M. hyorhinis* BTS-7 (X58555), *M. penetrans* GTU54 (D89508), *M. muris* RH14 (D89507), *M. pneumoniae* Mac (D14528), *M. genitalium* G37 (D14526), *M. fastidiosum* ATCC33229 (AY781782), *Ureaplasma urealyticum* T960 (X58561), *U. cati* F2 (D636859), *U. canigenitalium* D6P-C (D63684), *M. haemomuris* Shizuoka (AB080799), *M. haemofelis* Gandai2 (AB638408), and 'C. M. haemobos' Gandai1 (AB638407). Scale bar indicates the estimated evolutionary distance that was computed with CLUSTAL W [26] using neighbor-joining method [23]. Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications.

stem-loop domains were allocated in ITS of *M. haemofelis* and 'C. M. haemobos' (Fig. 3). The boxA was assigned on the stem portion of domain IV, and this was common to the structure of 'C. M. haemomuris' [8]. No identical architecture was evident between these two species. Domain III was seen in both the species in common, despite a single nucleotide substitution that was capable to form a stable secondary structure to minimize free energy, by G-T wobble as well as canonical Watson-Crick base pairings at stem region. Secondary structures in ITS region have sometime provided a key character to distinguish closely related species among mycoplasmas [6, 7, 9].

Here we demonstrated that ITS regions of hemoplasmas possess a feature of the genus *Mycoplasma*, and hemoplasmas form a monophyletic relationship with *M. fastidiosum* in phylogenetic analysis. A common stem-loop structure was identified in *M. haemofelis* and 'C. M. haemobos', though its significance remained unknown.

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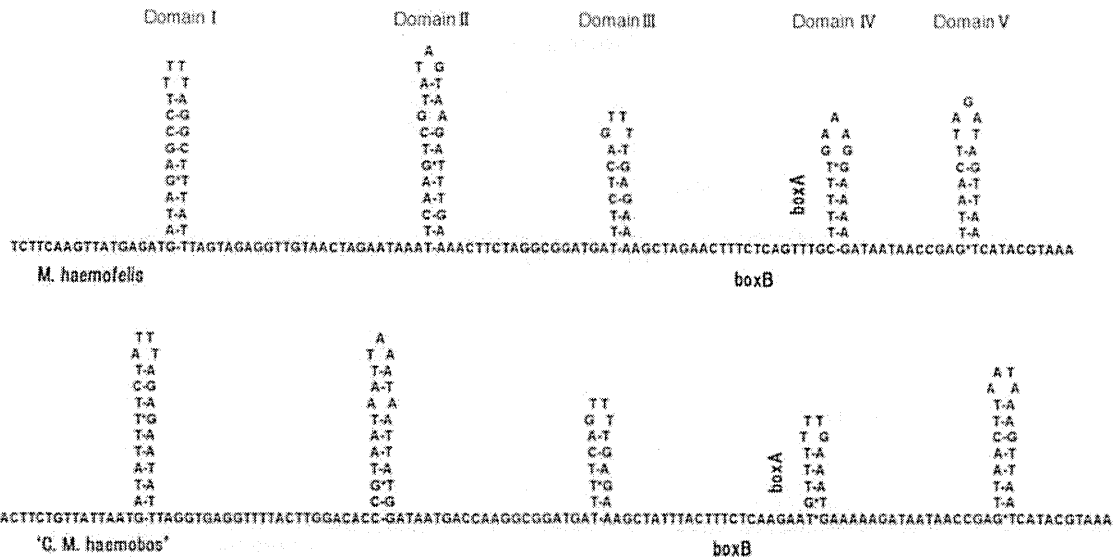


Fig. 3. Hypothetical secondary structures for the ITS of *M. haemofelis* (top) and '*C. M. haemobos*' (bottom). Canonical Watson-Crick base-pairing is hyphenated, and a wobble base-pairing tolerated in the secondary structure is shown by an asterisk. The boxA was a part of the stem region of domain IV, and boxB was located between domains III and IV.

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