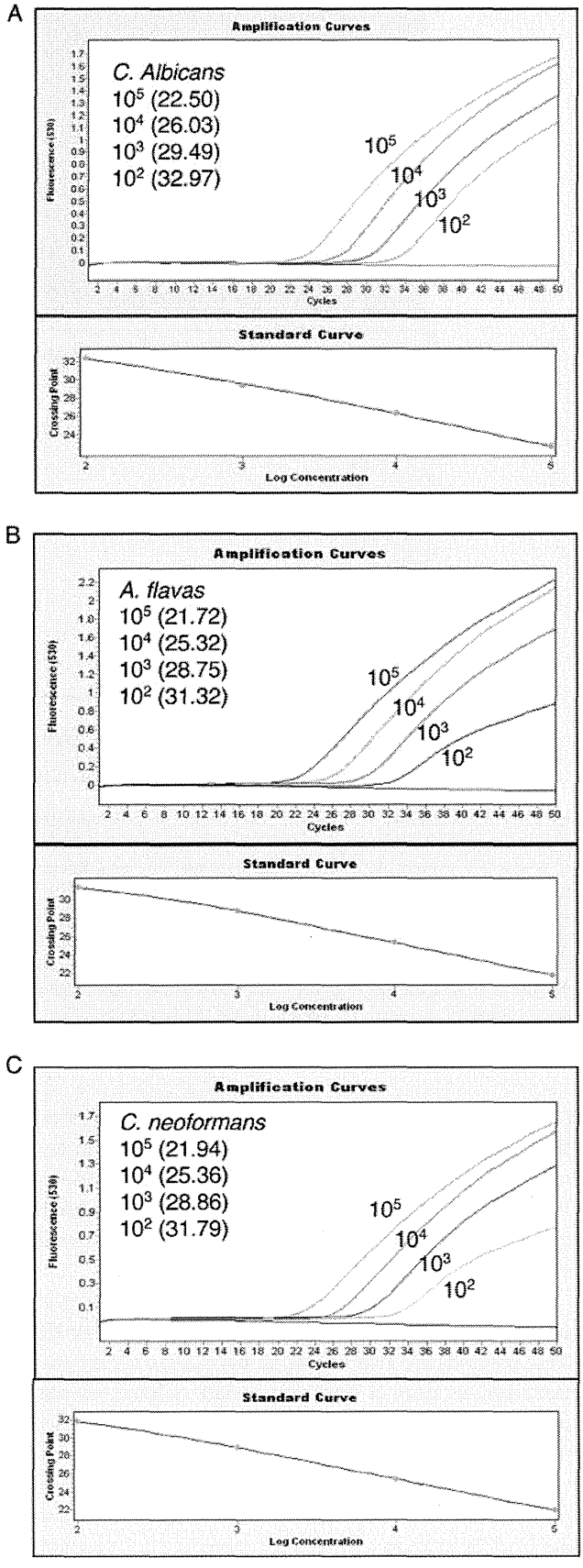

Fig. 1 PCR assay sensitivity. To examine the sensitivity of the broad-range real-time PCR for fungal 28 S rDNA, PCR fragments were amplified from DNA of *Candida* (*C. albicans,* **a**), *Aspergillus* (*A. flavas,* **b**), and *Cryptococcus* (*C. neoformans,* **c**) species. The *number in the parenthesis*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

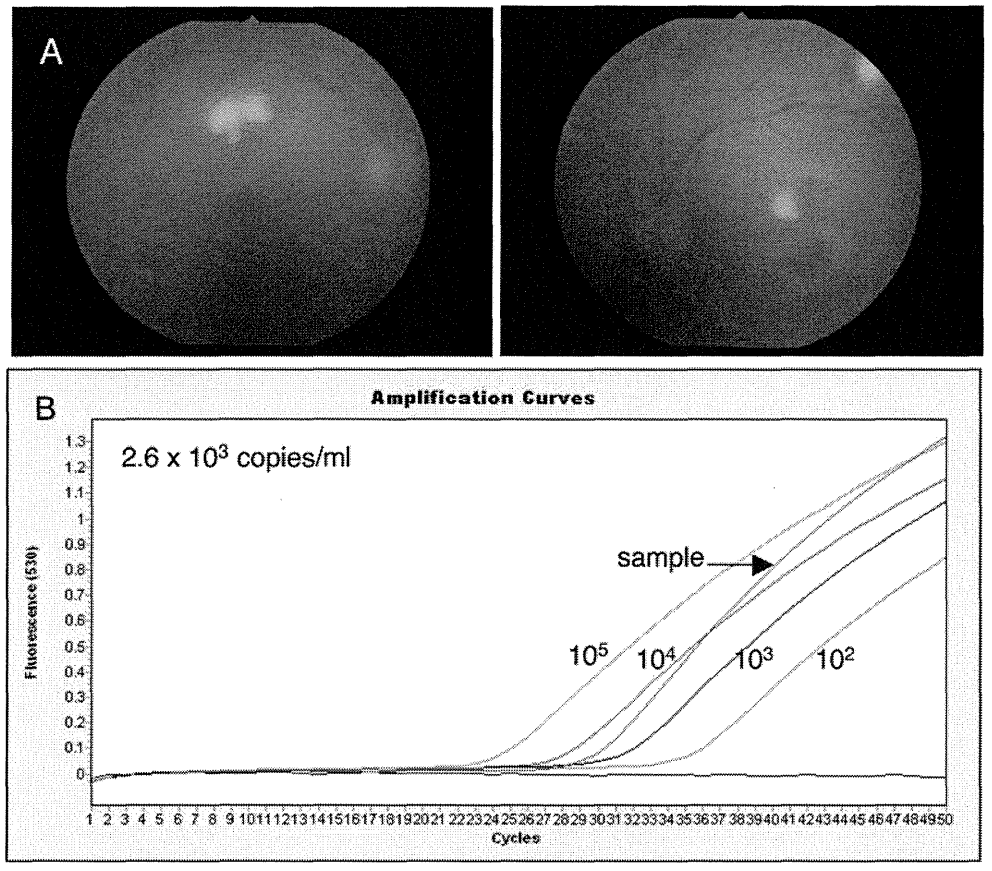

Fig. 2 A representative PCR result from a patient with endogenous endophthalmitis related to *Aspergillus* infection (case 179 in Table 1). A 74-year-old woman with type II diabetes mellitus was treated for splenic cancer. After surgery, she used intravascular catheters. She reported blurred vision and 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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Virological Analysis in Patients with Human Herpes Virus 6–Associated Ocular Inflammatory Disorders

Sunao Sugita,[1,2] *Norio Shimizu,*[3] *Ken Watanabe,*[3] *Manabu Ogawa,*[2] *Kazuichi Maruyama,*[4] *Norio Usui,*[5] *and Manabu Mochizuki*[2]

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 exanthema 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

From the [1]Laboratory for Retinal Regeneration, RIKEN Center for Developmental Biology, Kobe, Japan; Departments of [2]Ophthalmology & Visual Science and [3]Virology, Medical Research Institute, Tokyo Medical and Dental University Graduate School of Medicine and Dental Sciences, Tokyo, Japan; [4]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan; and [5]Department of Ophthalmology, Shinkawabashi Hospital, Kanagawa, Japan.

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Corresponding author: Sunao Sugita, Laboratory for Retinal Regeneration, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan; sunaoph@cdb.riken.jp.

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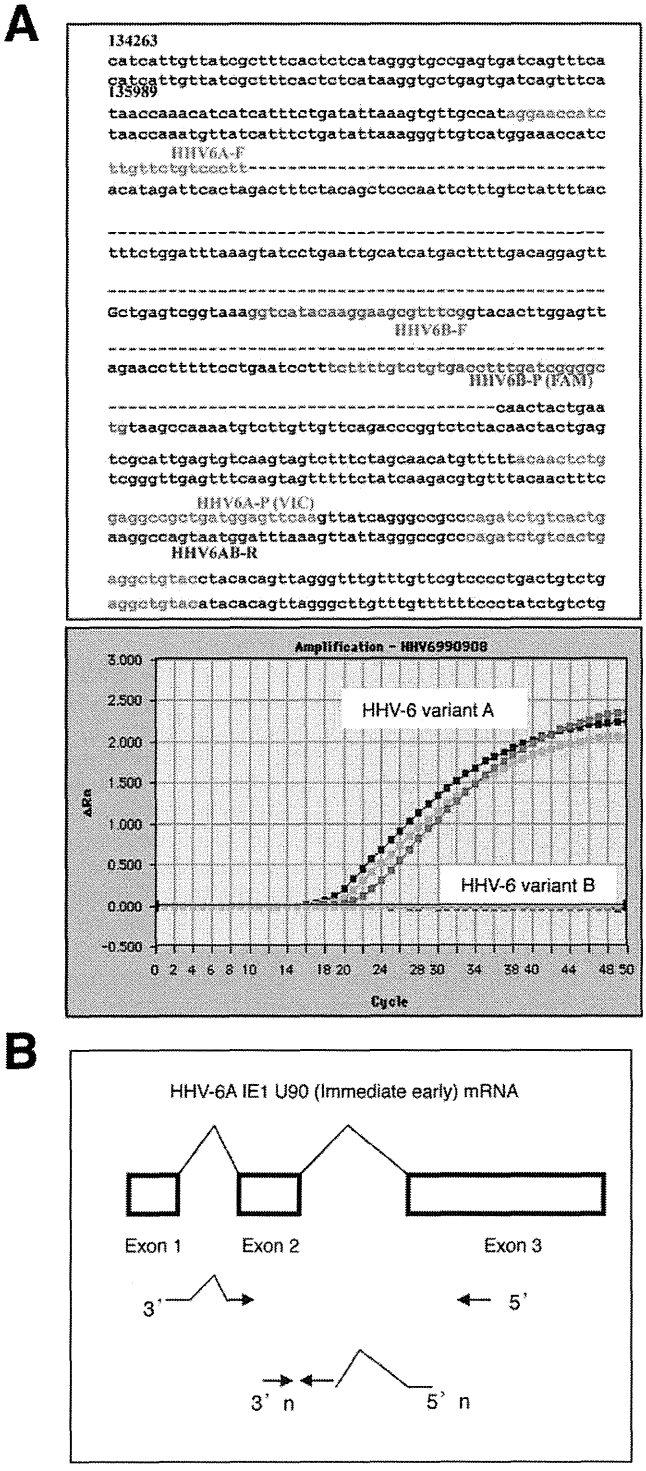

Figure 1. Amplification of HHV-6–specific DNA and mRNA. (**A**) TaqMan probes and primers used to amplify HHV-6 DNA (HHV-6A and HHV-6B). HHV-6 subtypes were identified using PCR with variant-specific primers and probes (*lower graph*). (**B**) Nested RT-PCR primers were designed to amplify HHV-6A mRNA.

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 trial 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	Cell1+	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	Karatitis	0.7	15	Infiltration	Cell1-	None	None	None	Culture (-) / PCR (+)	Bacterial karatitis

* 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: 2.0×10^4copies/mL, EBV: <50 copies/mL		
2	Aqh	HHV-6, HSV-1	HHV-6: 7.5×10^3copies/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/μg · DNA	HHV-6B	Antibiotics

Aqh, aqueous humor; IAI, intravitreal antibiotic injection; PPV, pars plana vitrectomy; PSL, prednisolone; SA, systemic antibitotics; 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, Rotkrevz, Switzerland). Primers for HHV-6 were as follows: Forward - ACCCGAGAGATGATTTTGCG and Reverse - GCAGAAGA-CAGCAGCGAGAT. Probes were used as follows: 3′FITC-TAAG-TAACCGTTTTCGTCCCA and LcRed705-5′-GGGTCATTTATGTTATAGA. 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 Amplitaq 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 AG-CAGCTGGCGAAAAGTGCTGTGC. 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 - CAGCGGACTGAGCAGCTA; nested PCR Forward - CCGATCCAATGATGGAAGAA and Reverse - CAGCGGACTGAGCAGC-TA (Fig. 1B). A one-step RT-PCR was performed on 100 ng of total RNA with 0.5 μM of each primer and SuperScript III One-Step RT-PCR with platinum Taq (Life Technologies Co., Tokyo, Japan) in a final volume of 50 μL. 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 μL of the first PCR solution using 0.5 μM 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 μg in a buffer containing 75 mM Tris-HCl (pH = 8.8), 0.01% Tween-20, 20 mM $(NH_4)_2SO_4$, and 1.5 mM $MgCl_2$ in a final volume of 50 μL. 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 μM each primer and SuperScript III One-Step RT-PCR with platinum Taq in a final volume of 50 μL (Forward-CTTCCTTCCTGGGCAT 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

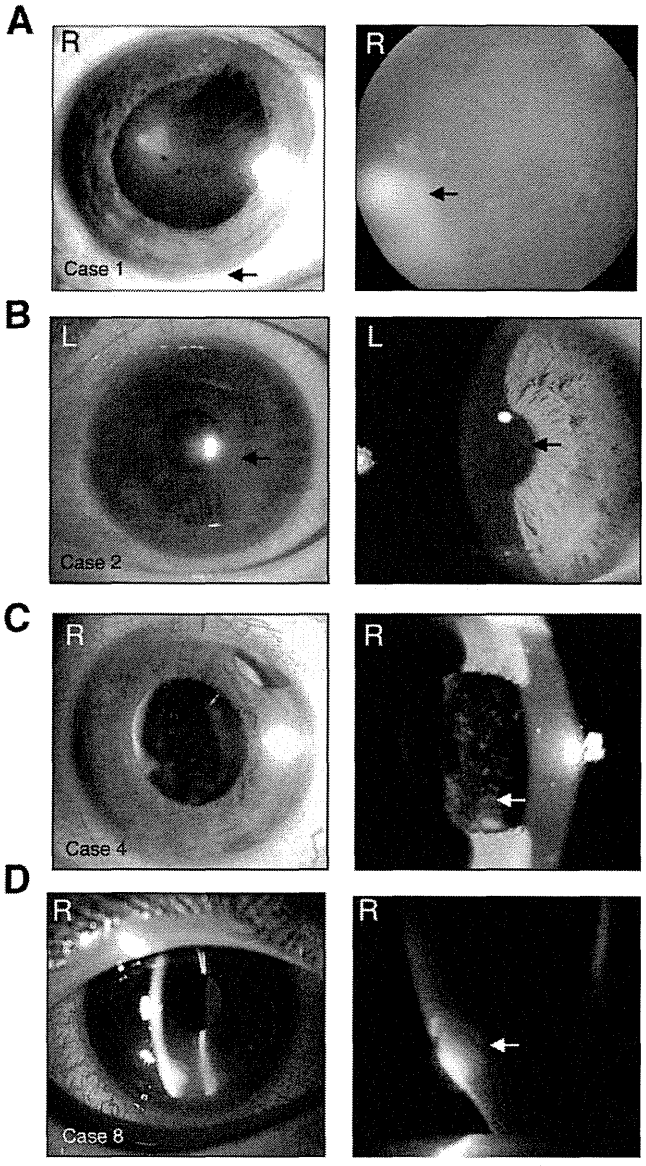

FIGURE 2. Slit-lamp and fundus photographs for HHV-6 infections. (**A**) Case 1: A case of ocular toxocariasis. Slit-lamp examination of right eye (RE) disclosed ciliary injection, moderate mutton-fat keratic precipitates (KPs), and severe anterior chamber cells with hypopyon (*arrow*). Funduscopic examination 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).[13] Distinguishing between HHV-6 subtypes is mainly accomplished using PCR techniques, including melting curve[19] 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

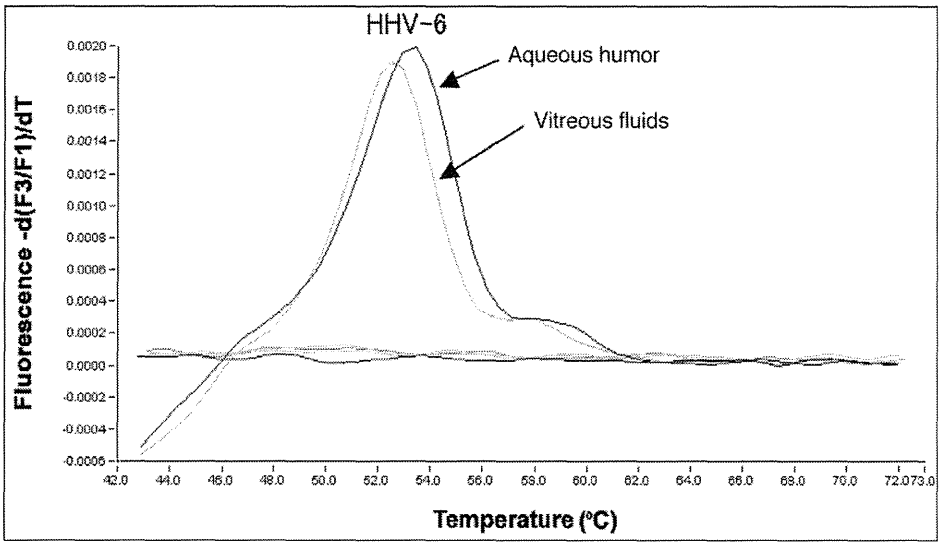

FIGURE 3. Results for multiplex PCR in a patient with HHV-6–positive uveitis. A significant positive curve was seen at 52°C, indicating detection of HHV-6 genomic DNA in the ocular fluids (case 1). DNA from other herpes viruses, such as HSV1, HSV2, VZV, EBV, CMV, HHV7, and HHV8, was not detected in this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

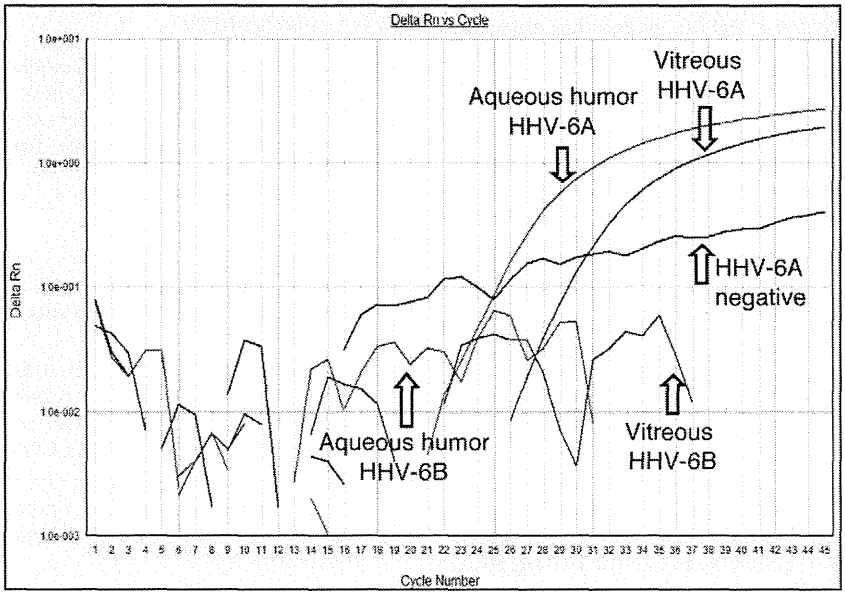

FIGURE 4. Detection of HHV-6 DNA by quantitative real-time PCR. The real-time PCR results for the samples from case 1 showed that intraocular samples, such as aqueous humor and vitreous fluids, contained a high copy number of HHV-6A DNA, but not HHV-6B DNA.

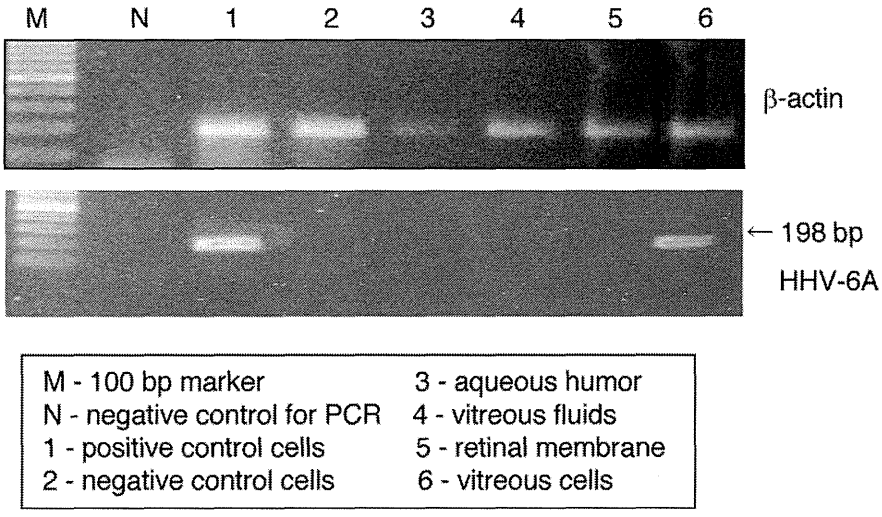

FIGURE 5. Detection of HHV-6 mRNA in intraocular samples. HHV-6A mRNA was detected in samples from vitreous cells, but other ocular samples, such as aqueous humor, vitreous fluids, and retinal membrane tissues were all negative (*lower image*). All samples, including control RNA, were positive for β-actin (*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 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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Kenji Nagata (Department of Ophthalmology, Kyoto Prefectural University of Medicine) and Yu Kaneko (Department of Ophthalmology, Yamagata University) collected and sent the samples used in this study. We are grateful for the expert technical assistance of Shizu Inoue.

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NOTE *Bacteriology*

Examination of the 16S-23S rRNA Intergenic Spacer Sequences of '*Candidatus* Mycoplasma haemobos' and *Mycoplasma haemofelis*

Fumina SASAOKA[1], Jin SUZUKI[1], Masatoshi FUJIHARA[1], Yusaku WATANABE[1], Kazuya NAGAI[2] and Ryô HARASAWA[1]*

[1]*Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka 020–8550, Japan*
[2]*Cryobiofrontier Research Center, Faculty of Agriculture, Iwate University, Morioka 020–8550, Japan*

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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 mycoplasms. 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'-GTTCCCAGGTCTTGTACACA-3') and reverse (5'-CAGTACTTGTTCACTATCGGTA-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 ethylenediaminetetracetate) 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/DDBJ 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,

* CORRESPONDENCE TO: HARASAWA, R., Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka 020–8550, Japan.
e-mail: harasawa-tky@umin.ac.jp

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

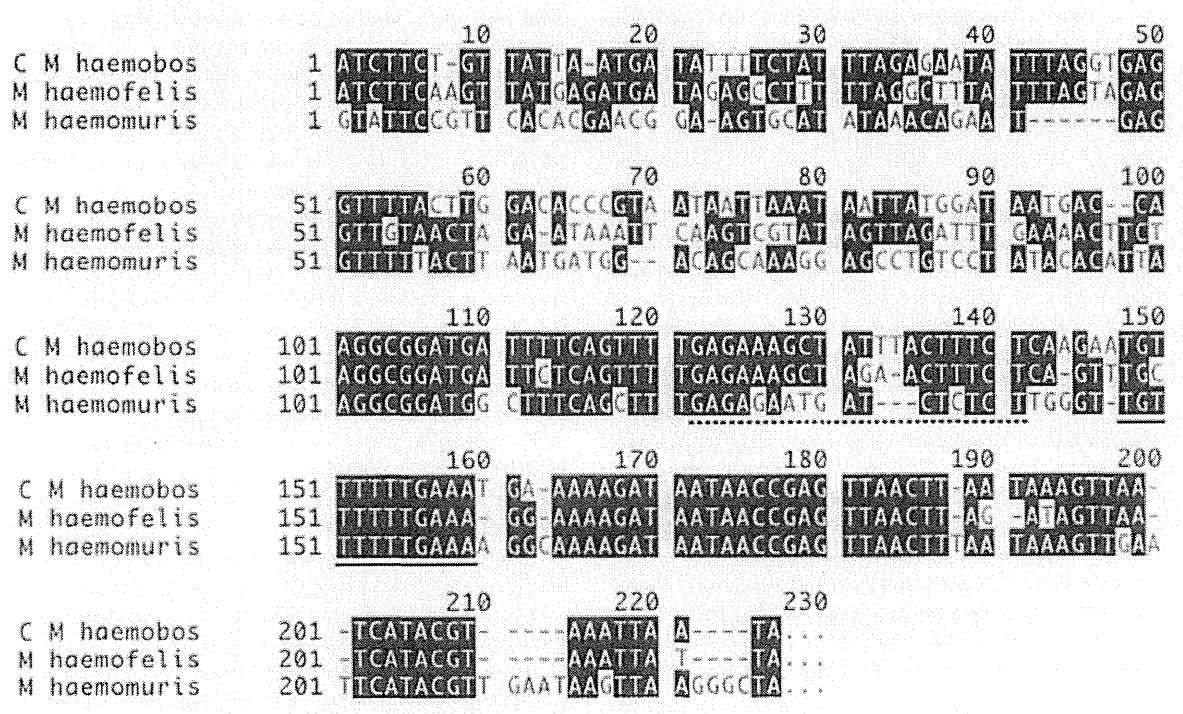

Fig. 1. Nucleotide sequence alignment of ITS from the three hemoplasma species, *M. haemofelis*, '*C.* M. haemobos' and *M. haemomuris*. The nucleotide sequence of *M. haemomuris* was obtained from the previous report [8]. Nucleotide sequence numbers are given from a consensus sequence. Homologous nucleotides 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.

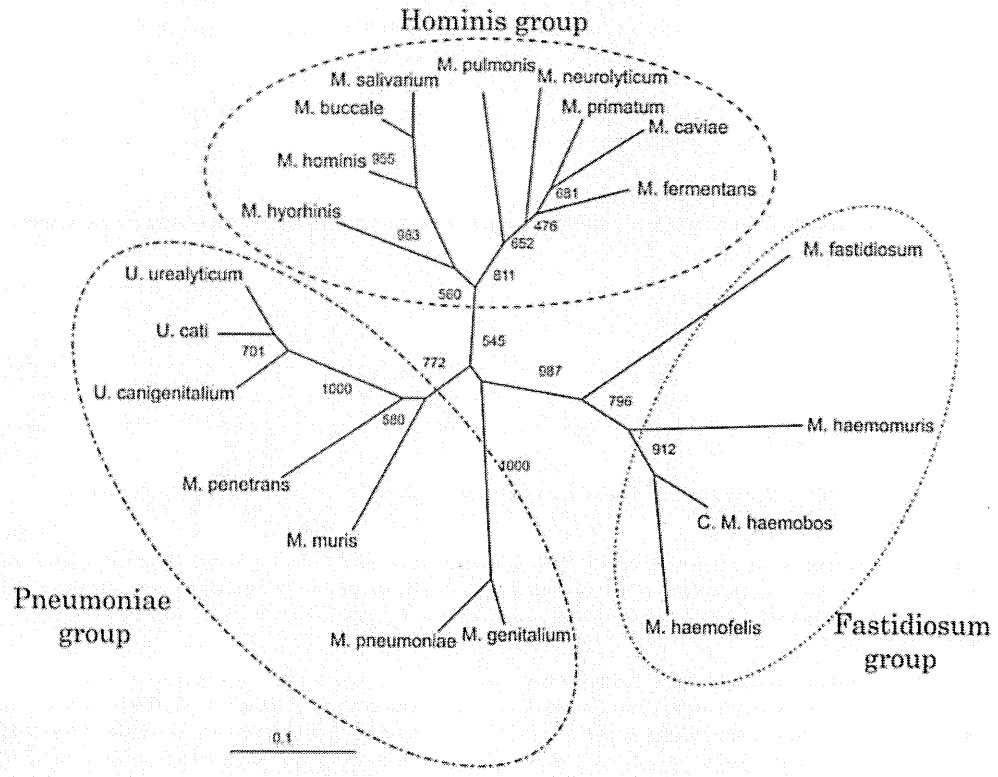

Fig. 2. Unrooted phylogenetic tree based on the ITS comparison, suggesting a monophyletic relationship among hemoplasmas and *M. fastidiosum*. Following nucleotide sequences were obtained from the DNA databases and accession number is given in parenthesis. They are *M. salivarium* PG20 (X58558), *M. 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. ppenetrans* GTU54 (D89508), *M. muris* RIII4 (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 posses a feature of the genus *Mycoplasma*, and hemoplasmas form a monophyletic relationship with *M. fastidioum* 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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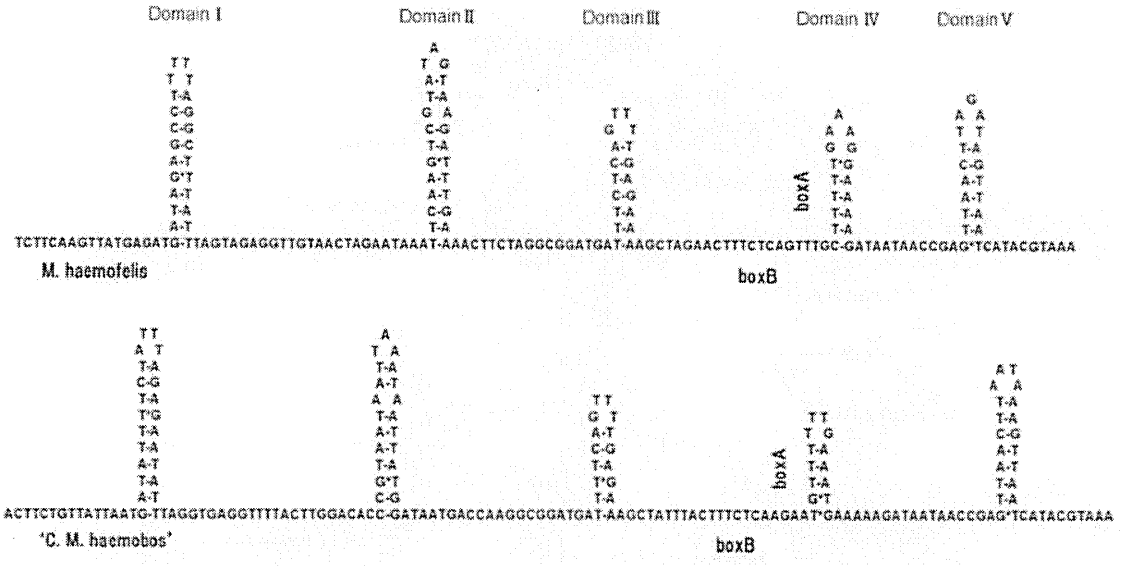

Fig. 3. Hypothetical secondary structures for the ITS of *M. haemofelis* (top) and '*C.* M. haemobos' (bottom). Canonical Watson-Crick base-pairing is hyphened, and a wobble base-pairing tolerated in the secondary structure is shown by an asterisk. The boxA was a part of the stem region of domain IV,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

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NOTE *Bacteriology*

Prevalence of Swine Hemoplasmas Revealed by Real-Time PCR Using 16S rRNA Gene Primers

Yusaku WATANABE[1,2,4], Masatoshi FUJIHARA[1,2], Jin SUZUKI[1], Fumina SASAOKA[1], Kazuya NAGAI[3] and Ryô HARASAWA[1,2]*

[1]*Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan*
[2]*Department of Applied Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Gifu 501-1193, Japan*
[3]*Cryobiofrontier Research Center, Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan*
[4]*Bremen Vet Center, Yahaba, Iwate 028-3602, Japan*

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ABSTRACT Hemoplasma is a tribal name for epierythrocytic mycoplasmas including *Mycoplasma suis* and *M. parvum* which are currently recognized in pigs as causative of porcine hemoplasmosis. Here, we report a real-time PCR assay for differential detection of these swine hemoplasma species by using allelic primers in the16S rRNA gene, and its application to survey for hemoplasma infections in pigs. Universal primers and species-specific primers were designed and evaluated by using swine blood samples positive in hemoplasmas. *Mycoplasma suis* and *M. parvum* infections were both confirmed by universal primers, and mixed infections were clearly distinguished by species-specific primers. Further, we applied this real-time PCR assay to 120 swine blood specimens from clinically healthy pigs in eleven farms in Japan, and found six (5.0%) were positive for *M. suis* and 18 (15.0%) were positive for *M. parvum*, and three (2.5%) were mixed infection by both hemoplasma species.
KEY WORDS hemoplasma, mycoplasma, swine.

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Hemoplasmas are epierythrocytic procaryotes that include mycoplasma species transferred from the *Eperythrozoon* and *Haemobartonella* genera, based on their similarity of the 16S rRNA sequences, and newly identified hemotropic mycoplasmas [2, 9]. They are uncultivable *in vitro* so far and causative of hemolytic anemia, resulting in icterus and pyrexia in various mammalian species. Two hemoplasma species, *Mycoplasma suis* and *M. parvum*, formerly *Eperythrozoon suis* and *E. parvum*, respectively, are currently known in pigs [14].

Mycoplasma suis is a causative agent of swine hemoplasmosis, previously called porcine eperythrozoonosis, of which symptoms are variable. In the acute form, anemia, icterus and anorexia are observed in sows [5], severe anemia and pyrexia in newborn and weaned piglets [2, 4]. Chronic infection following acute form may depress growth rate and increase susceptibility to other infectious diseases in feeder pigs, resulting to reproductive failures or immunosuppression in sows [4, 9, 18]. The clinical signs of *M. parvum* infection have not well been documented, despite severe anemia along with pyrexia in splenectomised pigs [1, 13].

Diagnosis of swine hemoplasmosis has been based on microscopic observation of the organisms on the surface of erythrocytes in Giemsa-stained blood smears, but this method is limited in acute infection, because the parasites are not always apparent unless the parasitemia is developed [10]. Serological tests including indirect hemagglutination (IHA) and enzyme-linked immunosorbent assay (ELISA) have also been applied for *M. suis* detection [8, 10], but they are not common because of difficulty in obtaining specific antigens. Polymerase chain reaction (PCR) using specific primers targeting the16S rRNA gene has most widely been used for screening various hemoplasmas [6, 12, 15–17]. Species identification of *M. suis* is usually depending on PCR [3, 7], but the identification of *M. parvum* has long been hampered due to lack of established strains maintained *in vivo* so far. This particular species was detected by real-time and end-point PCR from clinically healthy pigs in a commercial farm, and the nucleotide sequences of almost entire region of the16S rRNA and RNase P RNA genes were recently determined [17].

In the present study, we evaluated universal and species-specific primers for PCR to detect swine hemoplasmas by applying to six swine blood specimens, designated A through F, shown positive in hemoplasma infection in our previous study [17]. To evaluate the primers for real-time PCR, we examined three sets of primers, consisting of an universal primer set Pig 16S and two species-specific primer sets, Parvum 16S specific for *M. parvum* and Suis 16S specific for *M. suis* (Table 1). The six swine blood specimens infected with hemoplasmas were subjected to real-time PCR by using these primers as described previously [17].

All the six blood specimens were positive for hemoplasma infection by the real-time PCR using the universal Pig 16S primer set. The PCR products showed the melting

*CORRESPONDENCE TO: HARASAWA, R., Department of Veterinary Microbiology, Faculty of Agriculture, Iwate University, Morioka, Iwate 020–8550, Japan.
e-mail: harasawa-tky@umin.ac.jp

Table 1. Primer sets and sequences of primer used in real-time and end-point PCR assays

Primer set	Primer name	Primer sequence (5' to 3')	Position	size
Pig 16S	Pig 16S F	5'-ttt tag tgg caa acg ggc ga-3'	60–79**	247 bp
	Pig 16S R	5'-tca atc cca ttg cgg ctg tt-3'	287–306**	
Parvum 16S	Parvum 16S F	5'-aac aca tat tta act tgc tc-3'	100–119*	137 bp
	Parvum 16S R	5'-cat att cct att cat ccg cg-3'	217–236*	
Suis 16S	Suis 16S F	5'-aac gca tac tta act tac tt-3'	82–101**	138 bp
	Suis 16S R	5'-cat act cct att tac ccg ct-3'	200–219**	
Parvum 16S-2	Parvum 16S F	5'-aac aca tat tta act tgc tc-3'	100–119*	484 bp
	Hemo 16S R	5'-cct acg ctt cct tta cgc cc-3'	546–565**	
Suis 16S-2	Suis 16S F	5'-aac gca tac tta act tac tt-3'	82–101**	483 bp
	Hemo 16S R	5'-cct acg ctt cct tta cgc cc-3'	546–565**	

* Relative to the *M. parvum* Morioka 9 (AB610850) sequence. ** Relative to the *M. suis* Illinois (U88565) sequence.

temperature (*Tm*) at 84.62 ± 0.33°C in the melting experiments. Specimens A, B and C were positive in the real-time PCR using the species-specific Parvum 16S primer set, and the *Tm* was 80.38 ± 0.13°C. All the specimens except B were positive in the real-time PCR using species-specific Suis 16S primer set, with *Tm* at 81.36 ± 0.14°C (Fig. 1).

Then, we performed end-point PCR by using the same primer sets as real-time PCR. The PCR consisted of 5 μl of DNA solution, 5 μl of 10× Buffer for KOD-Plus-Ver.2, 5 μl of 2 mM dNTPs, 3 μl of 25 mM $MgSO_4$, 0.3 μl of forward primer, 0.3 μl of reverse primer (50 pmol/μl each), 1 μl of KOD -Plus- (1 U/μl) and water to a final volume of 50 μl. After the denaturation at 98°C for 2 min, the reaction was carried out 30 cycles with denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The amplified products were fractionated by 2.5% agarose gel electrophoresis with a Quick-Load 100 bp DNA Ladder marker (New England BioLabs Inc., Ipswich, MA, U.S.A.), and photographed by the GelDoc-It Imaging system (UVP, Upland, CA, U.S.A.) after staining with 0.4 μg/m*l* ethidium bromide.

By using the primer set Pig 16S, all the specimens produced a relevant sized band in the end-point PCR. In the case of Parvum 16S primer set, specimens A, B and C produced a relevant sized band, and in the case of Suis 16S primer set, specimens A, C, D, E and F produced a relevant sized band. All the end-point PCR results were consistent with real-time PCR (Fig. 2). In addition, the PCR products obtained by using species-specific primer sets were too small to determine the nucleotide sequence in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.).

Next, we explored two other primer sets, Parvum 16S-2 and Suis 16S-2, consisting of species-specific forward primers and a common universal reverse primer bracketing about 500 bp portion of the 16S rRNA gene (Table 1), by subjecting all the six blood specimens to end-point PCR as described previously [16]. The amplified products were fractionated by 1.0% agarose gel electrophoresis with a 200 bp ladder DNA size marker (TaKaRa Bio., Otsu, Japan), and photographed as described above.

By using the primer set Parvum 16S-2, specimens A, B and C produced a relevant sized band to *M. parvum* in the end-point PCR. In the case of the Suis 16S-2 primer set, specimens A, C, D, E and F produced a relevant sized band to *M. suis*, though the band of specimen A was faint to compare to others (Fig. 3). These PCR products were

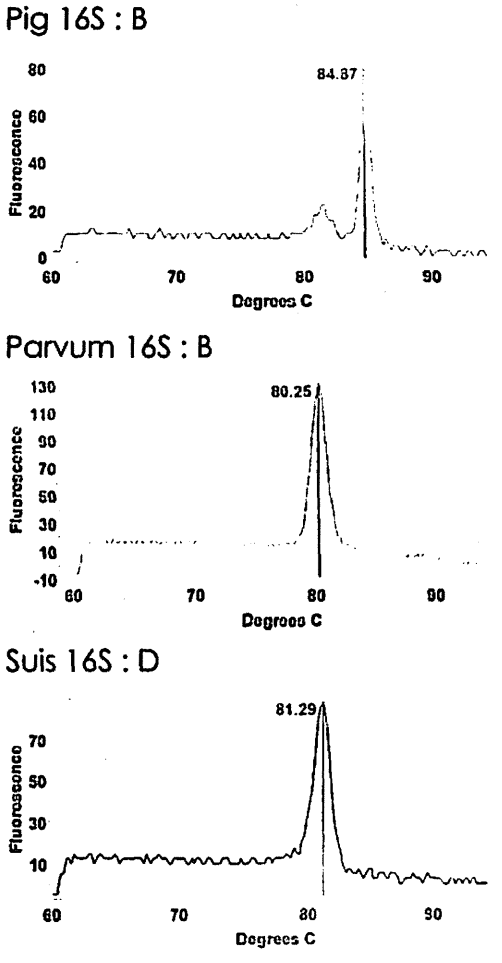

Fig. 1. Representative melting curves using primer sets Pig 16S (top: specimen B), Parvum 16S (middle: specimen B) and Suis 16S (bottom: specimen D).

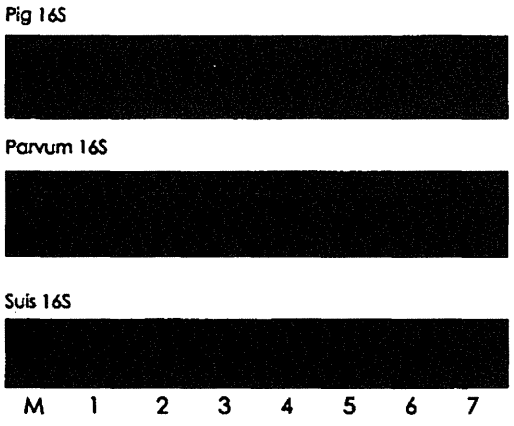

Fig. 2. Agarose gel electrophoresis of the PCR products amplified from six blood samples infected with hemoplasma using three primer sets, Pig 16S, Parvum 16S and Suis 16S. Lane M, DNA size marker (100 bp DNA ladder); lane 1, negative control; lane 2, A; lane 3, B; lane 4, C; lane 5, D 1; lane 6, E; lane 7, F.

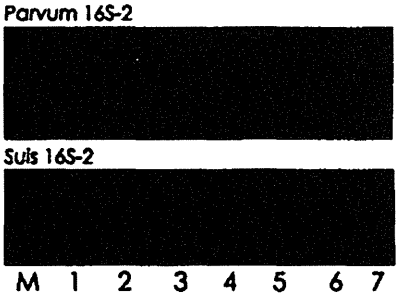

Fig. 3. Agarose gel electrophoresis of the PCR products amplified from six blood samples infected with hemoplasma using two primer sets, Parvum 16S-2 and Suis 16S-2. Lane M, DNA size marker (200 bp DNA ladder); lane 1, negative control; lane 2, A; lane 3, B; lane 4, C; lane 5, D 1; lane 6, E; lane 7, F.

further subjected to direct sequencing as described above. All the nucleotide sequences of PCR products obtained by using Parvum 16S-2 primer set were identical to *M. parvum* sequence. On the other hand, the nucleotide sequences of the PCR products obtained by using Suis 16S-2 were identified as *M. suis*, though sequencing of the specimen A was failed in our experiment. Taken together these results, existence of both *M. parvum* and *M. suis* was confirmed in specimen C, and this indicates the existence of mixed infection of both swine hemoplasmas that was not revealed by the universal primers. End-point PCR using Parvum 16S-2 and Suis 16S-2 primer sets were not sensitive as compared to real-time and end-point PCRs using Parvum 16S and Suis 16S primer sets for detection of swine hemoplasmas.

Our data support that the universal primers allow to detection of hemoplasma infection by real-time PCR as well as end-point PCR, but they were not able to distinguish the mixed infection of swine hemoplasmas.

Each hemoplasma species was detected by both real-time and end-point PCRs by using Parvum 16S or Suis 16S primer sets, but the real-time PCR with melting experiments was much more convenient than the end-point PCR. Thus, the real-time PCR using species-specific primers seemed more useful to evaluate the epidemiology of swine hemoplasmas.

Lastly, we applied real-time PCR in the same condition using species-specific primer sets to investigate the prevalence of swine hemoplasmas in 120 blood samples collected from clinically healthy pigs including 108 feeders and twelve sows raised in eleven different commercial swine farms in Japan. Of them, 18 (15.0%) pigs in six (54.5%) farms were positive for *M. parvum*, and six (5.0%) pigs in one (9.1%) farm were positive for *M. suis*. Mixed infections with both hemoplasma species were detected in three (2.5%) blood samples.

Our results first demonstrated the existence of mixed infection with *M. parvum* and *M. suis* in swine, and also revealed the prevalence of swine hemoplasma infections in Japanese farms nowadays. *Mycoplasma suis* infection causes panleucopenia in both piglets and sows and immunosuppression under the stressed conditions by parturition for sows or weaning for piglets [4]. This may lead to increased infections of respiratory and enteric diseases [6], and also it pertinent to consider this organisms affect as etiological agent with recent swine disease complex associated with emergent infectious diseases due to *Porcine circovirus 2* infection etc. Besides, human infection with *M. suis* has been reported among swine farm workers in China [18] and should be considered in aspects of public hygiene. Although *M. parvum* has been thought relatively less pathogenic to swine [13], a clinical episode has been recorded in Japanese commercial swine farm in 1984 [11]. Thus, an in-depth investigation of swine disease complex is extremely urgent, because of high incidence of hemoplasma infection in the modern swine operation of this country.

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