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H. 知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（障害者対策総合研究事業（感覚器障害分野））
（分担）研究報告書

難治性眼炎症性疾患に対する網羅的迅速診断システムの開発

研究分担者　東京医科歯科大学大学院医歯学総合研究科眼科学分野　講師
杉田　直

研究要旨：感染性ぶどう膜炎、眼内リンパ腫などの難治性眼炎症性疾患に対する網羅的迅速診断システムの開発を行い、その有効性について検討した。その結果、これらの眼疾患に対してこの検査は有効であった。

A．研究目的

PCR 法を用いて眼科炎症性疾患に関連する多くの病原微生物ゲノムを網羅的に微量の検体から検出することにより、疾患の診断をするシステムを開発し、そのシステムの有効性を検討する。

B．研究方法

臨床所見から眼内感染症、眼内リンパ腫が疑われた患者より眼検体を採取し全例で PCR 検査にてウイルス（ヘルペスウイルス属）、細菌（細菌全般 16S rDNA）、寄生虫（トキソプラズマ、トキソカラ）、真菌（カンジダ、アスペルギルス）などを調べた。また、臨床所見より必要症例に対し眼内リンパ腫の PCR を施行した。

（倫理面への配慮）

本研究はヘルシンキ宣言の趣旨を尊重し、学内倫理委員会の承認のもと患者のインフォード・コンセントを得た上で施行した。UMIN 臨床試験登録システムへの登録を行った。

C．研究結果

検査の結果、確定診断例は 142 例、感染除外例は 187 例、無効例は 31 例であった。その感度は 83%、特異度は 99%で、臨床所見から眼内感染症あるいは眼内リンパ腫が疑われる症例に対してこの網羅的迅速 PCR 診断システムは有効であった。

D．考察

当初の目的であった、PCR システムの構築および多数例の臨床検体での PCR システムの有効性の検証につき達成できた。PCR システムの感度、特異度は高く臨床の場で広く活用できるものであった。そのため、このシステムの導入により、眼科領域の感染症や悪性腫瘍に対し早期診断、早期治療が可能になると思われた。

E．結論

臨床所見から眼内感染症あるいは眼内リンパ腫が疑われる症例に対してこの網羅的迅速 PCR 診断システムは有効であった。

F．研究発表（平成 23 年度）

1．論文発表

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G. 知的財産権の出願・登録状況（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

厚生労働科学研究費補助金（障害者対策総合研究事業（感覚器障害分野））
（分担）研究報告書

難治性眼炎症性疾患に対する網羅的迅速診断システムの開発

研究分担者　大阪大学　眼科　中井　慶　助教

研究要旨
難治性眼炎症性疾患に対する網羅的迅速診断システムの開発

A．研究目的

難治性眼炎症性疾患に対する網羅的迅速診断システムの開発すること

B．研究方法

患者からの承諾のもと、前房水、硝子体液を回収し、医科歯科大学へ送付、その際、個人名を伏せて番号で送付。

そして、1ヶ月以内に患者番号と結果が当院に送付され、患者に診断を伝える。

（倫理面への配慮）

患者が同定されないように、すべて患者番号で取り扱いされる。

C．研究結果

今年度、約40例程度において、このシステムを利用したが、日常診療に非常に有用であった。

D．考察

このシステムは日常診療に非常に有用であると考えられる。

E．結論

このシステムは日常診療に非常に有用であると考えられる。

F．研究発表（平成23年度）

特になし

2．学会発表

特になし

G．知的財産権の出願・登録状況

（予定を含む。）

特になし

厚生労働科学研究費補助金（障害者対策総合研究事業（感覚器障害分野））
（分担）研究報告書

難治性眼炎症性疾患に対する網羅的迅速診断システムの開発

研究分担者　東京医科大学　眼科　助教　　臼井嘉彦

研究要旨：感染性ぶどう膜炎、眼内リンパ腫などの難治性眼炎症性疾患に対する網羅的迅速診断システムの開発を行い、その有効性について検討した。その結果、これらの眼疾患に対してこの検査は有効であった。

A．研究目的

難治性眼炎症性疾患に対する網羅的迅速診断システムを用いて、ぶどう膜炎における診断の向上につながるか否かを検討した。

B．研究方法

原因不明のぶどう膜炎患者の眼内液から DNA を抽出し、broad-range real-time PCR を施行した。

（倫理面への配慮）

本研究はヘルシンキ宣言の趣旨を尊重し、東京医科大学倫理委員会の承認のもと患者のインフォームド・コンセントを得た上で施行した。

C．研究結果＆D．考察

71 例中 28 例は感染性ぶどう膜炎（ヘルペス性虹彩毛様体炎、急性網膜壊死、真菌性眼内炎、細菌性眼内炎など）であることがわかった。

71 例中 43 例は、このシステムを用いて感染性ぶどう膜炎を除外できたことにより、ポスナーシュロスマン症候群、水晶体過敏性眼内炎、眼内悪性リンパ腫、フックス異色性虹彩毛様体炎などの診断にいたった。しかし、結核、トキソカラ、梅毒によるぶどう膜炎はこのシステムによりぶどう膜炎の診断にはいたらなかった。

E．結論

今回使用したいシステムにより、結核、トキソカラ、梅毒を除くほぼすべてのぶどう膜炎について診断することができた。

F．研究発表（平成 23 年度）

1．論文発表

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2) 臼井嘉彦、森秀樹、奥貫陽子、森地陽子、毛塚剛司、坂井潤一、後藤浩. 視力予後良好であった急性網膜壊死の臨床像の検討. 第 65 回日本臨床眼科学会　2011.10.7-10 東京

G．知的財産権の出願・登録状況

（予定を含む。）

1．特許取得

なし

2．実用新案登録

なし

3．その他

厚生労働科学研究費補助金（障害者対策総合研究事業（感覚器障害分野））
（分担）研究報告書

難治性眼炎症性疾患に対する網羅的迅速診断システムの開発

研究分担者　武田　篤信　九州大学大学院医学研究院眼科　助教

研究要旨
Polymerase chain reaction(PCR)法を用いて、眼内の微量検体より眼内感染症に関連する大部分の病原微生物、あるいは眼内リンパ腫を一度に診断もしくは除外できるシステムを構築し、そのシステムの有用性を検討する。

A．研究目的

Polymerase chain reaction(PCR)法を用いて、眼内の微量検体より眼内感染症に関連する大部分の病原微生物、あるいは眼内リンパ腫を一度に診断もしくは除外できるシステムを構築し、そのシステムの有用性を検討する。

B．研究方法

臨床所見から眼内感染症あるいは眼内リンパ腫が疑われた患者より眼検体を採取し前例でPCR検査にてウイルス(ヘルペスウイルス属)、細菌(細菌全般 16S rDNA)、寄生虫(トキソプラズマ、トキソカラ)、真菌(カンジダ、アスペルギルス)などを調べた。また、臨床所見より必要症例に対し眼内リンパ腫のPCRを施行した。

これらのウイルス、細菌、真菌の病原微生物の塗抹標本・培養の陽性率は高くなく、感染症診断のGold standardが現状では存在しない。そのため、症例を次の3つの項目に分けてシステムの有効性を検討した。即ち、(1)確定診断例：PCRの結果が陽性となり臨床所見と一致し確定診断に至ったもの。(2)感染症除外診断例：PCRの全項目陰性で臨床所見、他の検査と一致し眼感染症、眼内リンパ腫が除外できたもの。(3)無効例：PCRの結果が臨床所見、他の検査と一致しないものである。

(倫理面への配慮)

本研究はヘルシンキ宣言の趣旨を尊重し、学内倫理委員会の承認のもと患者のインフォームド・コンセントを得た上で施行した。

C．D．研究結果及び考察

承認後これまでに43名、46例に対しPCR検査を行った結果、確定診断例は眼内悪性リンパ腫6例、感染性5例であった。感染症除外例は24例、無効例は11例であった。臨床所見から眼内悪性リンパ腫、眼内感染症が疑われる症例に対して、この網羅的迅速PCR診断システムは有効である可能性があると考えられた。今後症例を集めてさらなる有効性を検討する予定である。

E．結論

臨床所見から眼内リンパ腫または眼内感染症が疑われる症例に対して、この網羅的迅速PCR診断システムは有効と考えられた。

F．研究発表(平成23年度)

1．論文発表

該当なし。

2．学会発表

該当なし。

G．知的財産権の出願・登録状況

（予定を含む。）

1．特許取得

該当なし。

2．実用新案登録

該当なし。

3．その他

厚生労働科学研究費補助金（障害者対策総合研究事業（感覚器障害分野））
（分担）研究報告書

難治性眼炎症性疾患に対する網羅的迅速診断システムの開発

研究分担　丸山　和一　京都府立医科大学 眼科 後期専攻医

研究要旨
Polymerase Chain Reaction（PCR）法を用いて、眼内の微量検体より眼感染症に関する大部分の病原微生物、あるいは眼内リンパ腫を一度に診断もしくは除外できるシステムを構築し、そのシステムの臨床的有効性を検討する。

A．研究目的

ウィルス・細菌 Broad-rangePCR の有効性の検討とともに、感染性眼内炎または非感染性眼内炎の判別を施行する事で臨床での治療に役立てることが可能かどうかを検討した。

B．研究方法

臨床所見より眼内感染症あるいは眼内悪性リンパ腫が疑われた患者またはサルコイドーシス患者より検体（網膜・硝子体液または前房水）を採取し、全例でPCR検査にてウィルス（ヘルペス属）、細菌、寄生虫、真菌などを判定した。また悪性リンパ腫を疑う症例では、PCR 検査を用いモノクロナリティを判定した。

これまで、硝子体液からサルコイドーシス発症に関与すると考えられていた *Propionobacterium acnes*（*P. acnes*）の DNA 検出は硝子体サンプルからの報告はほとんど無いため、本研究結果は合致するかどうかを検討した。

本研究はヘルシンキ宣言の趣旨を尊重し、当大学内倫理委員会の承認のもと患者のインフォームドコンセントを得た上で施行した。

C．研究結果

PCR 検査の結果、現在までの報告通りサルコイドーシス内眼炎患者硝子体内からは *P. acnes DNA* は検出されなかった。しかし、肺組織生検と同様に肉芽腫形成のある網膜組織からは *P. acnes DNA* が高コピー数検出された。また硝子体液で細菌培養が陽性であった細菌性眼内炎・臨床的に急性網膜壊死と考えられた症例では、培養結果と同様に全例で細菌 16sDNA・ヘルペス DNA が高コピー検出された。

D．考察

臨床所見から眼内感染症が疑われる症例に対して網羅的迅速PCR診断システムが有効で有り、且つ PCR 検査結果が陰性と考えられる疾患では陰性の結果が得られた。このためステロイドなどの抗炎症治療（免疫抑制）が早期から使用できることも今後症例を増加し、本診断法の有効性を検討する予定である。

E．結論

臨床所見から眼内感染症が疑われる症例／否定出来る症例に対してこの網羅的迅速 PCR 診断は有効であった。

F．研究発表（平成 23 年度）

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4) 丸山和一, 永田健児, 小嶋健太郎, 安原　徹, 米田一仁, 杉田　直, 望月　學, 稲葉　亨, 木下　茂. 高齢者サルコイドーシス内眼炎における硝子体液のフローサイトメトリー解析. 第 11 回日本抗加齢学会総会. 2011.05.27. 京都

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G. 知的財産権の出願・登録状況

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Sugita S, Kamoi K, Ogawa M, Watanabe K, Shimizu N, Mochizuki M.	Detection of candida and Aspergillus species DNA using broad-range real-time PCR for fungal endophthalmitis.	Graefes Arch Clin Exp Ophthalmol	In press		2011
Imadome K, Yajima M, Arai A, Nakazawa A, Kawano F, Ichikawa S, Shimizu N, Yamamoto N, Morio T, Ohga S, Nakamura H, Ito M, Miura O, Komano J, Fujiwara S.	Novel mouse xenograft models reveal a critical role of CD4+ T cells in the proliferation of EBV-infected T and NK cells.	PLOS Pathogenes	7	1-14	2011
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CLINICAL INVESTIGATION

Diagnosis of ocular toxoplasmosis by two polymerase chain reaction (PCR) examinations: qualitative multiplex and quantitative real-time

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Abstract
Aim To establish a two-step polymerase chain reaction (PCR) diagnostic system for ocular toxoplasmosis.
Methods A total of 13 ocular fluid samples (11 aqueous humor and 2 vitreous fluid) were collected from 13 patients with clinically suspected ocular toxoplasmosis. Ten ocular samples from other uveitis patients and 20 samples from subjects without ocular inflammation were used as controls. Two polymerase chain reaction (PCR) methods, i.e., qualitative multiplex PCR and quantitative real-time PCR, were used to measure the toxoplasma genome (*T. gondii* B1 gene).
Results Qualitative multiplex PCR detected *T. gondii* B1 gene in the ocular fluids of 11 out of 13 patients with clinically suspected ocular toxoplasmosis. In real-time PCR, we detected high copy numbers of *T. gondii* DNA (5.1×10^2–2.1×10^6 copies/mL) in a total of 10 patients (10/13, 77%). Only ocular toxoplasmosis scar lesions were observed in the three real-time PCR-negative patients. PCR assay results for the samples from the two control groups were all negative.
Conclusions The two-step PCR examination to detect toxoplasma DNA is a useful tool for diagnosing ocular toxoplasmosis.

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Keywords Ocular toxoplasmosis · Polymerase chain reaction · Uveitis · Ocular fluids

Introduction

Ocular toxoplasmosis is a sight-threatening intraocular inflammatory disorder prevalent in many parts of the world. In clinical practice, ocular toxoplasmosis diagnosis is made based on *Toxoplasma gondii* (*T. gondii*) serological tests and on the findings of typical ocular manifestations, for example old retinal necrotic lesions with pigmentation and fresh retinal lesions adjacent to chorioretinal atrophic lesions. However, there are many asymptomatic sero-positive individuals in the area in which *T. gondii* is endemic, with atypical lesions of ocular toxoplasmosis that resemble other necrotizing retinitis, for example acute retinal necrosis and cytomegalovirus retinitis. It is, therefore, necessary to perform laboratory tests to confirm toxoplasmosis infections in the eye. Ocular fluids, which include the aqueous humor and vitreous fluid, are ideal samples for this test, because they can be used to examine local specific antibody production (Goldmann–Witmer coefficient; GWC) or *T. gondii* DNA by polymerase chain reaction (PCR). Previous reports reveal that GWC and PCR assays performed on ocular samples can play a prominent role in the diagnosis of *Toxoplasma* infections [1–12]. Because local specific antibody production is often unpredictable in immunocompromised patients, the PCR assay is reported to be a better diagnostic tool [10]. In addition, the PCR assay can also be used to examine ocular samples for the purpose of diagnosing ocular toxoplasmosis in immunocompetent patients [11] and the atypical strain of *T. gondii* [12]. Moreover, previous studies found that PCR is a rapid and sensitive method

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for detecting *T. gondii* quantitatively in clinical specimens [13–16]. However, no previous studies have screened other pathogenic agents that could cause necrotizing retinitis in conjunction with *T. gondii*.

In this study, we attempted to measure the *Toxoplasma* genome in ocular samples of patients with clinically suspected ocular *Toxoplasma* by using a two-step PCR system with specific primers and probes for *T. gondii* DNA amplification (*T. gondii* B1 gene). To screen for the human herpes virus and *T. gondii*, the first step used qualitative multiplex PCR to detect the toxoplasma genome in the ocular sample. In the second step, quantitative real-time PCR was used to measure the genomic DNA of *T. gondii*.

Materials and methods

Subjects

This research followed the tenets of the Declaration of Helsinki, with the study protocol approved by the Institutional Ethics Committee of Tokyo Medical and Dental University. Ocular fluid samples were collected only after each patient had provided written informed consent.

Table 1 summarizes the clinical findings observed for patients with ocular toxoplasmosis at their initial presentation. The first patient group was examined between January 2008 and September 2010 at the Tokyo Medical and Dental University Hospital. This group included 13 consecutive patients clinically suspected of having ocular toxoplasmosis based on the serological test for *T. gondii* (serum anti-Toxo IgG: PHA method) and characteristic ocular manifestations. Of these 13 patients, 10 had active intraocular inflammation, that is, there were anterior chamber cells, vitreous opacity, retinal vasculitis, and fresh retinal exudates (focal retinal necrosis). For the other 3 patients, only inactive ocular toxoplasmosis lesions in the form of old pigmented retinal scars were found. For the PCR assay, we collected intraocular fluids from 13 patients (11 aqueous humor and 2 vitreous fluids).

In the second group, we collected 10 samples (8 aqueous humor and 2 vitreous fluid) from 10 patients with other clinical entities of uveitis. The diagnoses for the subjects included idiopathic uveitis ($n = 7$), acute retinal necrosis ($n = 2$), and cytomegalovirus retinitis ($n = 1$). At the time of sampling, all members of this group had active intraocular inflammation.

In the third group, we collected 20 samples (15 aqueous humor and 5 vitreous fluid) from 20 patients with non-inflammatory diseases. The patient diagnoses included age-related cataract ($n = 15$), primary rhegmatogenous retinal detachments ($n = 1$), idiopathic macular hole ($n = 1$), and idiopathic epiretinal membranes ($n = 3$).

The sampling procedures were performed in accordance with the method reported in our previous studies [17–19]. Briefly, we used surgical microscopy to aseptically collect aliquots of approximately 0.1 ml aqueous humor in a syringe with a 30 G needle. Non-diluted vitreous fluid (approximately 0.5 ml) was collected during the pars plana vitrectomy.

Polymerase chain reaction

DNA was extracted from samples by use of a DNA Mini Kit (Qiagen, Valencia, CA, USA) installed on a robotic workstation for automated purification of nucleic acids (BioRobot E21, Qiagen). For the DNA extraction, approximately 0.1 ml aqueous humor and 0.2 ml vitreous fluid were used. DNA was eluted with 60 µl elution buffer, the amount of DNA used for PCR was 5 µl.

For the PCR assay, we used standard toxoplasma DNA strains for the *T. gondii* RH strains. To detect the toxoplasma genome (*T. gondii* B1 gene), we used two PCR assays, the qualitative multiplex PCR and the quantitative real-time PCR. Multiplex PCR was designed to qualitatively detect genomic DNA of human herpes viruses, i.e., herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), and human herpes virus type 6 (HHV6), type 7 (HHV7), and type 8 (HHV8). PCR was performed using a LightCycler (Roche, Basel, Switzerland). Primers and probes of HHV1-8 and the PCR conditions have been described elsewhere [17, 18]. In addition to the herpes virus PCR, we calibrated the primers and the probe for detecting toxoplasma DNA (*T. gondii* B1 gene) as shown in Table 2. Specific primers for the virus were used with AccuPrime Taq (Invitrogen, Carlsbad, CA, USA). Products were subjected to 40 cycles of PCR amplification. Hybridization probes were then mixed with the PCR products. Real-time PCR was only performed for *T. gondii* when the genomic DNA of *T. gondii* was detected by multiplex screening PCR.

The real-time PCR was performed using AmpliTaq Gold and the Real-Time PCR 7300 system (Applied Biosystems, Foster City, CA, USA). The PCR conditions used for the *T. gondii* B1 gene were: 95°C for 0 s and 60°C for 20 s for 50 cycles. The PCR conditions used for the human herpes viruses have been described elsewhere [17, 18]. When more than 10 copies/mL were detected, the sample copy number was regarded as significant.

Results

Figure 1 shows representative PCR data (Case 1, Table 3). The multiplex PCR performed in order to screen all 8

Table 1 Clinical findings at initial presentation for patients with ocular toxoplasmosis

Case	Age	Sex	Eye	Initial findings and inflammation of AC					Duration of the symptoms	Vitreitis	Retinal vasculitis	Retinal exudates	
				VA	IOP (mmHg)	Granulomatous KPs	AC: cell	AC: flare				Old	Fresh
1	58	M	L	0.1	19	+	3+	131	2 months	+	+	+	+
2	70	F	L	0.3	21	+	2+	76	3 weeks	+	−	+	+
3	68	F	R	0.8	14	−	2+	34	2 months	+	+	−	+
4	44	M	R	1.0	12	+	1+	17	1.5 months	+	−	+	+
5	56	M	L	0.7	22	+	2+	43	3 weeks	+	+	+	+
6	65	F	L	1.0	15	+	1+	26	2 weeks	+	−	−	+
7	48	M	R	0.4	14	+	3+	124	3 weeks	+	+	+	+
8	35	M	L	0.6	18	−	1+	14	1 month	+	+	+	+
9	49	M	L	0.9	18	+	2+	29	1 month	+	+	+	+
10	59	F	R	0.5	20	−	1+	23	1.5 months	+	−	+	+
11	47	M	R	1.2	17	−	−	8	None	−	−	+	−
12	53	F	L	1.2	13	−	−	12	None	−	−	+	−
13	71	M	R	0.9	16	−	−	11	None	−	−	+	−

All patients were immunocompetent. "Old retinal exudates" indicates inactive ocular toxoplasmosis lesions in the form of old pigmented retinal scars

VA visual acuity, *IOP* intraocular pressure, *KPs* keratic precipitates, *AC* anterior chamber

Table 2 Design of primers and probe for detecting toxoplasma DNA (*T. gondii* B1 gene)

For multiplex PCR (qualitative PCR)
Primer F—TCCCCTCTGCTGGCGAAAAGT
Primer R—AGCGTTCGTGGTCAACTATCGATTG
LCRed640—GGTGTATTCGCAGATTGGTCGCCTG-P
Probe—CGAAAAGTGAAATTCATGAGTATCTGTG CAACT-6FAM
For Real-time PCR (quantitative PCR)
Primer F—TCCCCTCTGCTGGCGAAAAGT
Primer R—AGCGTTCGTGGTCAACTATCGATTG
Probe—6FAM-TCTGTGCAACTTTGGTGTATTCGCAG-iowaBK

We designed the primers and probes for the multiplex PCR and real-time PCR. The design of the primers is the same for the two PCR methods, although the relative positions of the TaqMan probe in the B1 gene were changed

human herpes virus DNAs and the *T. gondii* DNAs were positive for the *T. gondii* DNA (Fig. 1a). However, this sample was negative for all human herpes virus DNA tests. In addition, quantitative real-time PCR revealed that there were 1.1×10^6 copies/mL of *T. gondii* DNA in this specimen (Fig. 1b). Figure 2 shows the ocular findings for the patient. At the initial presentation, we made a clinical diagnosis of ocular toxoplasmosis based on both the clinical features and the serological tests (serum anti-Toxo IgG: ×640). Based on these findings, we treated the patient with systemic acetylspiramycin and prednisolone for 3 months. The treatment was effective and the active ocular lesions in the left eye completely disappeared. Two months after the treatment, a subsequent PCR indicated that the *T. gondii* DNA in the aqueous humor sample was now undetectable.

Table 3 summarizes the PCR results. Qualitative multiplex PCR for the *T. gondii* B1 gene was positive for 11 out of 13 patients with clinically suspected ocular toxoplasmosis (Table 3). Real-time PCR detected the B1 gene but not the human herpes virus DNA in the 10 patients who were clinically suspected of having ocular toxoplasmosis (10/13, 77%). In addition, high copy numbers of *T. gondii* DNA were detected (5.1×10^2–2.1×10^6 copies/mL) in all of these 10 patients, with active ocular inflammatory lesions that were compatible with ocular toxoplasmosis, i.e., focal retinal necrosis, vitreous opacity, anterior chamber cells, and choroidal edema with possible old scars. The only factors in the three PCR-negative patients that were compatible with an ocular toxoplasmosis diagnosis were the inactive scar lesions, i.e., old pigmented retinal scars. Of note is the finding that in one of these three patients *T. gondii* DNA was detected by the multiplex qualitative PCR in the aqueous humor sample (Case 12 in Table 3), even though the real-time PCR showed negative results (<10 copies/mL). A fundus photograph of a patient with inactive ocular toxoplasmosis is seen in Fig. 3. For this particular patient (Case 11 in Table 3), the PCR results

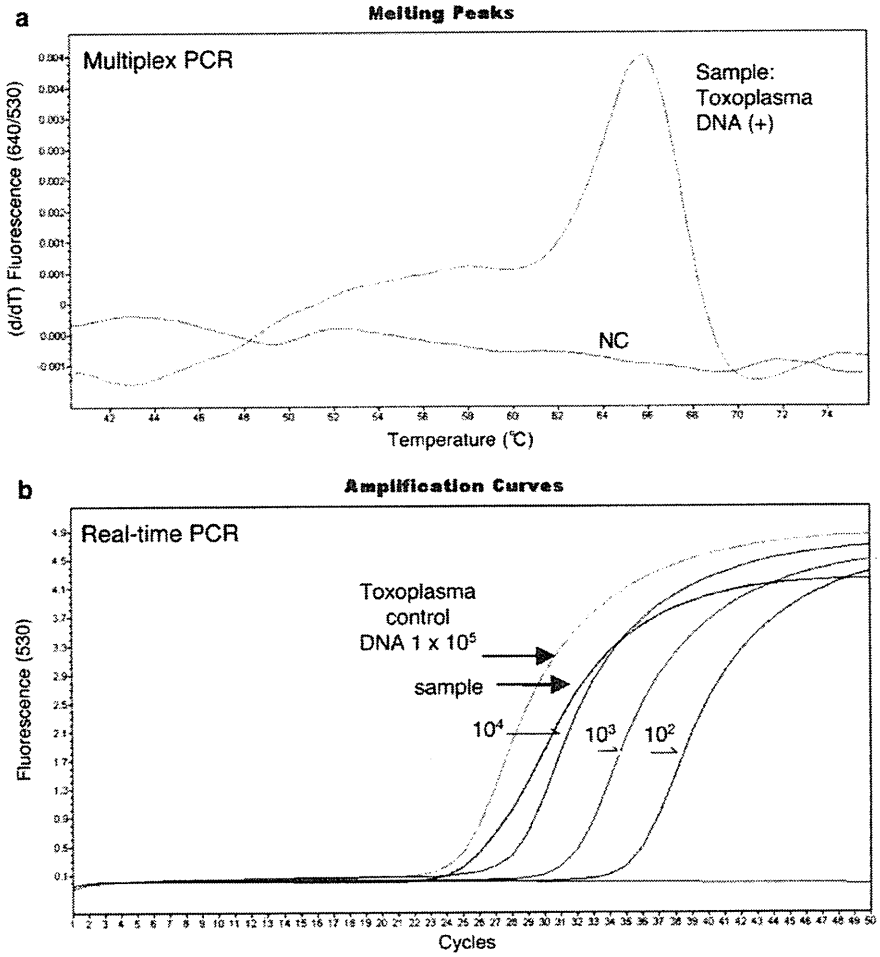

Fig. 1 PCR results for a patient with ocular toxoplasmosis (Case 1 in Table 3). **a** After DNA extraction from the sample, multiplex PCR was performed to screen for *T. gondii*, and for HHV1 to HHV8 using LightCycler capillaries. At 66°C, a significant positive curve was detected, indicating the detection of *T. gondii* genomic DNA in the aqueous humor. Using other LightCycler capillaries, human herpes viruses HSV1, HSV2, VZV, EBV, CMV, HHV6, HHV7, and HHV8 were negative for this sample. The flat line indicates the negative control. **b** Quantitative real-time PCR of the same sample shown in **a**. We calculated the copy number of the *T. gondii* genomic DNA in the sample. We measured both the tested ocular sample and control DNA (10^5, 10^4, 10^3, and 10^2 copies/mL) by real-time PCR, and then established the standard curve using the results of the control DNA. The standard curve was used to calculate the DNA concentration for the cycle threshold (*Ct*) value of the sample. The final copy number of genomic DNA in the sample (copies/mL) was calculated on the basis of the obtained sample volume and final dilution volume. Values were regarded as significant when more than 10 copies/mL were observed. The real-time PCR revealed there were 1.1×10^6 copies/mL of *T. gondii* DNA in this analyzed sample

were negative. In the serum of all of the ocular toxoplasmosis patients, the anti-toxoplasma IgG was positive (Table 3).

Negative PCR results were obtained for all the control uveitis patient samples (Cases 14–23 in Table 3) and for the control non-uveitis patients (data not shown).

Discussion

Using intraocular fluids for PCR gene amplification is helpful in diagnosing various ocular diseases, because it is possible to detect an exceedingly small amount of nucleic acid in a small ocular sample volume with high sensitivity. We report here a new PCR assay system that uses two separate steps, multiplex screening PCR and quantitative real-time PCR. With this new system, it becomes possible to detect *T. gondii* and rule out human herpes virus-related necrotizing retinitis. For these two PCR analyses in this study, oligonucleotide primers and a TaqMan probe were designed to amplify the *T. gondii* B1 gene. Our results clearly demonstrate that the PCR assay system succeeded in detecting the *T. gondii* DNA in the ocular fluid samples of the 10 patients with active ocular toxoplasmosis lesions,

Table 3 Detection of *T. gondii* DNA by qualitative multiplex PCR and quantitative real-time PCR in ocular samples from clinically suspected ocular toxoplasmosis

Case	Disease	Sample	Multiplex PCR	Real-time PCR (copies/mL)	Serum anti-Toxo IgG	Treatment
1	Toxoplasmosis (active)	AH	*T. gondii* DNA+	*T. gondii* DNA: 1.1×10^6	640	ASPM, PSL
2	Toxoplasmosis (active)	AH	*T. gondii* DNA+	*T. gondii* DNA: 1.6×10^3	320	ASPM
3	Toxoplasmosis (active)	AH	*T. gondii* DNA+	*T. gondii* DNA: 5.1×10^2	640	ASPM, PSL
4	Toxoplasmosis (active)	AH	*T. gondii* DNA+	*T. gondii* DNA: 3.0×10^4	2560	ASPM, PSL
5	Toxoplasmosis (active)	AH	*T. gondii* DNA+	*T. gondii* DNA: 9.4×10^4	5120	ASPM, PSL
6	Toxoplasmosis (active)	AH	*T. gondii* DNA+	*T. gondii* DNA: 5.5×10^4	2560	ASPM, PSL
7	Toxoplasmosis (active)	AH	*T. gondii* DNA+	*T. gondii* DNA: 9.9×10^2	640	CLDM
8	Toxoplasmosis (active)	VF	*T. gondii* DNA+	*T. gondii* DNA: 1.1×10^4	640	ASPM, PSL, PPV
9	Toxoplasmosis (active)	AH	*T. gondii* DNA+	*T. gondii* DNA: 4.2×10^3	2560	ASPM, PSL
10	Toxoplasmosis (active)	VF	*T. gondii* DNA+	*T. gondii* DNA: 2.1×10^6	1280	ASPM, PSL, PPV
11	Toxoplasmosis (old)	AH	–	<10	2560	None
12	Toxoplasmosis (old)	AH	*T. gondii* DNA+	<10	320	CLDM
13	Toxoplasmosis (old)	AH	–	<10	640	None
14	Idiopathic uveitis	AH	–	<10	320	None
15	Acute retinal necrosis	VF	VZV DNA+	VZV DNA: 8.3×10^6	<160	Valaciclovir, PPV, PSL
16	CMV retinitis	AH	CMV DNA+	CMV DNA: 9.0×10^5	<160	Ganciclovir
17	Idiopathic uveitis	AH	–	<10	<160	None
18	Idiopathic uveitis	VF	–	<10	<160	PSL, PPV
19	Idiopathic uveitis	AH	–	<10	<160	None
20	Acute retinal necrosis	AH	VZV DNA+	VZV DNA: 9.9×10^5	<160	Valaciclovir, PSL
21	Idiopathic uveitis	AH	–	<10	<160	PSL
22	Idiopathic uveitis	AH	–	<10	1280	PSL
23	Idiopathic uveitis	AH	–	<10	<160	None

We performed two PCR examinations using qualitative multiplex PCR and quantitative real-time PCR. Qualitative multiplex PCR was performed to screen for detection of the DNA of human herpes virus (HHV1-HHV8) and *T. gondii*. All samples from ocular toxoplasmosis (Cases 1–13) were negative for HHV-DNA. Anti-toxoplasma IgG was positive in the serum of all ocular toxoplasmosis patients. We collected a second ocular sample from cases 1, 2, 6, 7, 8, and 10, and performed PCR examinations. The results were all negative for DNA of human herpes virus and *T. gondii*

AH aqueous humor, *ASPM* acetylspiramycin, *CLDM* clindamycin, *PPV* pars plana vitrectomy, *PSL* prednisolone, *VF* vitreous fluids

but not in the three samples with inactive lesions. In addition, PCR did not detect any of the human herpes virus DNAs in any of the samples, nor did these PCR methods detect *T. gondii* DNA in any of the control patients. These results therefore suggest that when intraocular fluid samples are examined by a sequence of multiplex PCR and real-time PCR, the results can be used to diagnose ocular toxoplasmosis.

In this study, there was one case (Case 12 in Table 3) for which the results were positive when using qualitative multiplex PCR and negative when using quantitative real-time PCR. The qualitative PCR examination is extremely sensitive and, as such, is able to detect DNA released from inactive parasites. This may be the reason for the discrepancy seen between the qualitative and quantitative assays. However, because the amounts of intraocular DNA are so low in such patients, these situations can be regarded as innocuous. Thus, when attempting to diagnose patients, both qualitative PCR and quantitative real-time PCR should be performed to ensure that any positive results are a result of active disease and not related to older non-active lesions. When using real-time PCR, we found there was a correlation between the high DNA loads in the ocular fluids, which translates as a high copy number of *T. gondii* DNA, and the intraocular inflammation in the uveitis patients with ocular toxoplasmosis. In fact, the case that was positive when using qualitative PCR and negative when using real-time PCR (Case 12) turned out to be a patient with inactive uveitis (old pigmented retinal exudates without inflammatory signs). In this particular case, before determining the actual reason for the positivity, we did administer clindamycin to the patient in order to prevent any possible recurrence.

Although both the Goldmann–Witmer coefficient (GWC) and PCR are useful for clinical specimen analyses [1–16] and can achieve similar levels of assay sensitivity,

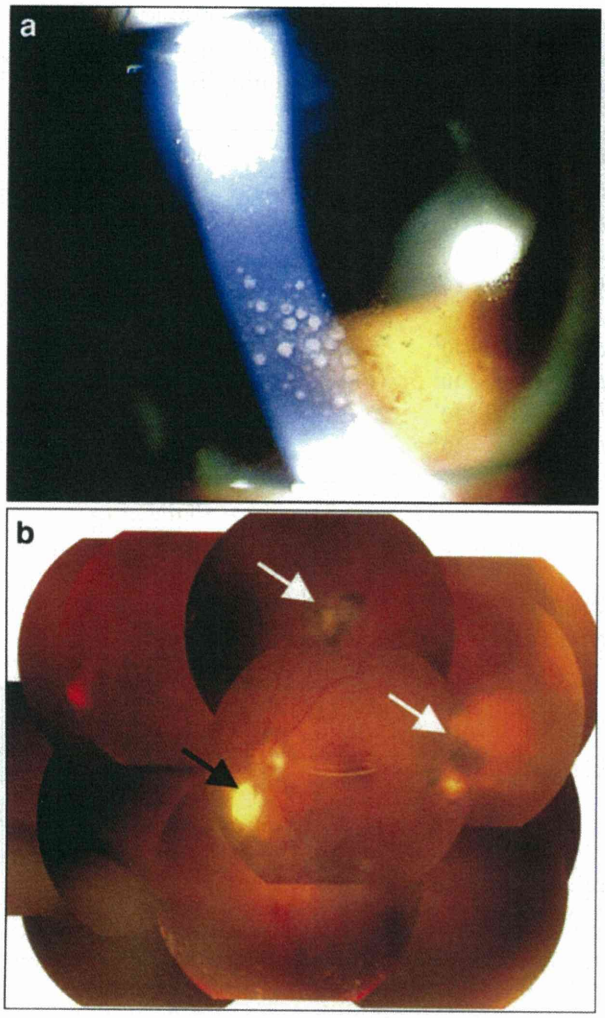

Fig. 2 Fundus and slit photograph of a patient with ocular toxoplasmosis (Case 1 in Table 3). **a** Slit and fundus photograph. **b** OS of a patient with an active toxoplasmosis infection. Diffuse keratic precipitates and anterior chamber cells (*upper panel*), and retinal yellowish white mass lesions (Edmund–Jensen type: *black arrow*) and retinal-pigmented exudates (*white arrows*) together with vitreous opacities are seen (*lower panel*)

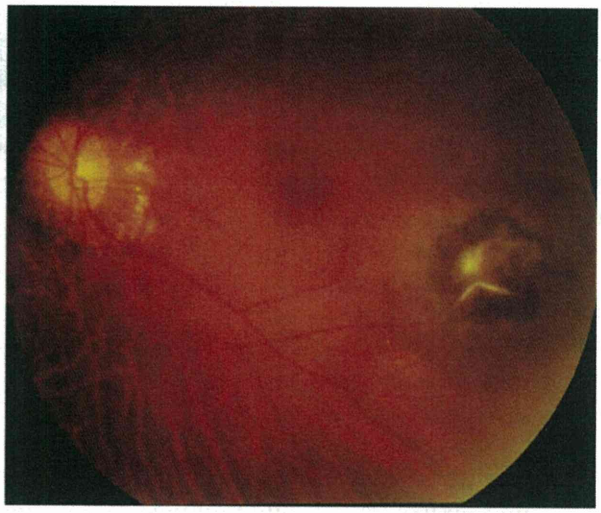

Fig. 3 A fundus photograph OS from an inactive ocular toxoplasmosis patient (Case 11 in Table 3). Old pigmented retinal exudates without inflammatory signs (vitreous cells, vitreous opacity, or retinal vasculitis) can be seen. PCR assay results were negative for genomic DNA of *T. gondii*

the proposed PCR system may be more advantageous since it has the ability to quantify the infection load of a clinical specimen. In addition, PCR examinations can exclude other major ocular infections that are caused by the human herpes virus. Westeneng et al. [7] reported 10 cases of ocular toxoplasmosis in immunocompromised patients. The PCR results were initially negative in 6 of these patients, with diagnosis only confirmed after use of the GWC. On the other hand, de Boer et al. report the use of PCR analysis was preferred for immunocompromised patients, because production of the local specific antibodies can be unpredictable in such patients [10]. Although the use of either PCR or GWC to diagnose ocular toxoplasmosis remains controversial, we were able to use PCR to detect the genomic DNA of toxoplasmosis in our immunocompetent patients even when they only had an active ocular inflammation. Therefore, this PCR methodology may be useful for *T. gondii* infection screening when used in conjunction with other diagnostic techniques, for example routine serological tests. In this study, we found increased anti-toxoplasma IgG in the serum of all of the ocular toxoplasmosis patients. However, we also found increased anti-toxoplasma IgG in the serum of two of our uveitis patients without ocular toxoplasmosis (Cases 14 and 22 in Table 3). We therefore recommend that PCR also be used to measure the toxoplasma DNA in ocular samples.

The protozoan parasite *T. gondii* has emerged as an important opportunistic infectious pathogen. In the eye, *T. gondii* infections can cause granulomatous pan-uveitis and necrotic retinitis, with typical ocular inflammation indicative of focal retinal necrosis, vitreous opacity, anterior chamber cells, and choroidal edema. Fundus lesions seen in ocular toxoplasmosis can be atypical in many patients, resembling necrotizing retinitis caused by human herpes viruses. The new PCR method is particularly useful when screening those uveitis patients who usually fail to generate specific IgM or increased IgG titers for *T. gondii* or who have had focal retinal necrosis. Thus, these results can be used to distinguish the findings from other retinal necrotic disorders, for example acute retinal necrosis and cytomegalovirus retinitis. By using several different primer pairs in LightCycler capillaries, these methods proved capable of rapidly screening for detection of the genome of

all eight types of human herpes virus and *T. gondii*. Development of this multiplex and real-time PCR assay seems to be quite advantageous, because this methodology makes it possible to exclude non-toxoplasma uveitis patients.

In conclusion, we have established a rapid, sensitive, comprehensive, two-step PCR system that can be used to detect *T. gondii*. New studies that examine larger numbers of samples from suspected ocular toxoplasmosis patients will need to be undertaken in the future in order to definitively establish the clinical value of this new diagnostic technique.

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INFLAMMATORY DISORDERS

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

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

Keywords Endophthalmitis · Fungal infection · Polymerase chain reaction

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Introduction

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

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

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

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

Methods

Sample preparation

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

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

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

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

Polymerase chain reaction

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

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

Results

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

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