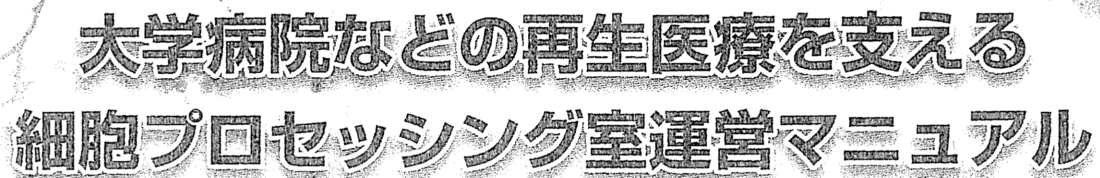

大学病院などの再生医療を支える
細胞プロセッシング室運営マニュアル

＊少人数の培養作業者で、効率よく、かつ安全性を確保した製造を目指して＊

対　象：

① 大学で再生医療を始める医師・歯科医師

② 再生医療の管理運営に関与する管理者

③ 培養作業者のGMPに準拠した教育訓練用

装　幀：A4　144ページ

執筆者：井上典子、梶昌美、小神晴美、渡辺真理、関根優、白山早起、藤本陽子（以上CPC所属）、
瀧澤淳、牧口智夫、布施一郎、中田光

監　修：森尾友宏、畠賢一郎、中田光

監修にあたって

再生医療が夢の治療として登場してから久しい。しかし、医療に広く再生医療が普及し、国が投じた予算が国民に還元されたかというと、残念ながら、まだ多くは研究段階のままである。培養細胞が製品として上市されたのは、株式会社JTECの培養皮膚シート、ジェイスだけである。再生医療産業は、1兆円規模の潜在的市場であるといわれているが、製造が承認され、保険収載までに開発に要する年月と投資額を考えると、大きなリスクのある産業分野といわざるを得ない。期待できるのは、大学病院など、臨床研究として再生医療の臨床開発ができる医療現場の細胞加工技術の向上である。平成21年度厚生労働省医療制度改革の中で、再生医療の普及は病院間連携・共同診療で進めていくという方針が決められ、今後、再生医療は、細胞プロセッシングセンターを持つ機関を中心に普及していくことが期待される。

そのときに問題となるのは、再生医療を実施、運営していく組織をどのように立ち上げるか、細胞加工のための設備をどのように造り、維持するのか、細胞製品の製造のための文書をどのように作成するのか、培養作業者をどのように教育するのかといった実務上のノウハウである。このノウハウは、Good Manufacturing Practice（GMP）と呼ばれる医薬品、医療機器、化粧品などの製造には欠かせない概念に準拠している。Good Clinical Practice（GCP）は治験に参加している多くの大学病院の医療従事者にとって馴染みやすい概念であるが、GMPの概念は、これまで大学病院などでは、あまり重視されてこなかった。GMPに関する成書は多いが、いざ、再生医療におけるGMPとは何かとなると難解で、既に細胞プロセッシングセンターを立ち上げて運営している施設からノウハウを学ばない限り、独自にシステムを立ち上げるのは困難であった。それは、GMPに準拠して、どのように再生医療のハード面、ソフト面を立ち上げるかということを解説した教科書が、これまでなかったからだろう。

本書は、最先端の培養技術や理論の紹介を目的としない。本書の目的は、実臨床としての再生医療を大学病院で始めようとする医療関係者を対象とし、具体的かつ実用的な情報を提供しようとするものである。また、細胞プロセッシングセンターを持たない施設の方々にもわかりやすいように豊富に図表と写真を用い、抽象的な表現を避け、具体例を提示して、可能な限りわかりやすい表現を心がけた。再生医療の現場で働く若手スタッフの教科書としても役立つものと思う。再生医療の研究には流行があると思うが、本書に記された内容には、普遍性があると信じ今後長きにわたって活用されることを願ってやまない。

最後に、お忙しいところを執筆していただいた著者の方々、貴重な情報をいただいた企業の方々、ご校閲いただいた株式会社デンカ生研青木恭二様、大塚浩史様に深くお礼申し上げます。

監　修
森尾　友宏
畠　賢一郎
中田　　光

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緒　言

どうやって細胞プロセッシングセンター（CPC）を立ち上げたか――その苦難な行程

初めて細胞プロセッシングセンター（Cell Processing Center:以下CPC）を設置する大学、病院などの読者のために、新潟大学医歯学総合病院で実際どうやってCPCを立ち上げたか、その経緯を紹介する。

2003年４月、新潟大学医歯学総合病院はトランスレーショナルリサーチの拠点として生命科学医療センターを新設し、その一部門として再生移植部門（後に輸血・再生医療部門に改名）を設け、従来の輸血部を改組してこれにあてることを決した。輸血部はそれ以前から、骨髄細胞、末梢血幹細胞などの採取や処理など細胞プロセッシングを必要としない細胞治療（骨髄移植、末梢血幹細胞移植など）を行っていたことから、院内の部門では最も適当と判断されたためである。

この決定を受け、我々はCPCを設立するためのプラニングを行うことになったが、院内の各診療科でばらばらの基準で行われていた細胞治療や再生医療を厚労省が推奨する基準に準じて安全に行うには、どういう構造の部屋を作り、どういう機器類が必要で、どのような組織作りが必要かなどを調査する、いわば暗中模索の時期がしばらく続いた。

その後、我々がまず手がけたのは、院内で細胞治療や再生医療を行っている、あるいは行うことを予定している各診療科の医師達に働きかけ、２週間に１度の割合でヒアリングを行うことであった。中には、まだ動物実験段階のものも含まれていたが、最終的に歯周病再生医療（培養骨膜シート）、血管新生療法（培養赤芽球）、口腔癌術後再生療法（培養口腔粘膜）が当面最も実用化に近い再生医療であることがわかり、この３プロジェクトをサポートするためのGMP準拠の細胞プロセッシング室の設置を目指すこととした。

(1)　設備と機器の立ち上げ

2005年11月、再生移植ワーキンググループを立ち上げて、院内の再生医療関係者に集まってもらい、部屋の設計、機器類などについての話し合いを開始した。部屋の設計に関しては、日本医科器械など細胞プロセッシング室の施工を手がけるいくつかの業者に見積りを依頼したが、高価であること、2006年１月に薬剤部が移転し、IVHの点滴詰めに使用されていたクリーンルームが空くことから、最終的にこの部屋を改装して使用することとなった。細胞プロセッシング用にHEPAフィルターを増設し、水道などの水回りや排水口などを撤去し、新たに前室と更衣室を設けた簡易型の細胞プロセッシング室が完成したのが2006年５月であった。ただ、この改築した部屋が本当にGMPに準拠した構造なのか、不備がないかなどの確信が得られなかったことから、同年７月に某社に施設性能検査と施設気密検査を依頼した。その結果、particle counterを用いた空気清浄度測定やフィルタリークの有無等の施設性能検査には問題がなかったものの、前室、更衣室のドアや、天井点検口、壁の電源アウトレットボックスなど多くの部位で気密不良が発見され、後日これらを密閉する作業を行わざるを得なかった。そして、後述する機器類を部屋へ搬入した後、同年９月に、今度は別の業者に同様の施設性能検査と気密検査を依頼し、浮遊微粒子数、浮遊細菌数ともクラス10,000を維持していること、気密性にも問題はないとの報告を受け、ようやく施設面については自信を得ることができたのである。

一方、細胞プロセッシング室の改装と併行して、上記3プロジェクトを行ううえで必要な機器類の選択作業を既に開始していた。各診療科の意見も聞き、最終的に下記のものを必要と判断した。

要求物品名	設置場所
マルチガスインキュベーター	BCR
炭酸ガス培養器	
位相差倒立顕微鏡（デジカメ付）	
位相差蛍光倒立顕微鏡	
血液バッグ用遠心機	
冷却マイクロ遠心機	
テーブルトップ型遠心機	
薬用保冷庫	
試験管ミキサー	
超低温フリーザー（－80℃）	細胞保存室
超低温フリーザー（－150℃）	
オートクレーブ（高圧蒸気滅菌器）	
超純水製造装置	
液体窒素保存容器	
製氷機	
遺伝子増幅装置	
機器監視システム	
中央実験台	
振とう器	
振とう恒温槽	
バーコード発行システム	
作業台	
殺菌ロッカー	更衣室

この機器要求を2006年2月に病院側に提出したところ、ヒアリングの後、幸い承認されたが、あとで実際に購入できるのは入札などがあるため1年後だということがわかり、部屋があっても中は空っぽという状態になりかけた。ここで我々の計画は頓挫しかけたが、わらにもすがる思いで応募した競争的資金が、幸いなことに2006年6月に大型予算に採択されたことで息を吹き返し、同年7月に主だった必要機器類を揃えることができた。

(2) 運転資金の調達

細胞プロセッシング室は完成し、必要な機器類も整備できたところで、次に我々の頭を悩ませたのは運転資金であった。病院は施設工事費、機器類の購入費は負担してくれたが、細胞培養を行うテクニシャン（培養作業者）の給料は1名分のみ、施設の維持費や消耗品などは自前でというスタンスであった。誠に理不尽なことと今でも思っているが、背に腹は代えられない。外部資金を導入する方向で検討を開始し、最終的に新潟市の企業・法人6社からなる「新潟再生医療コンソーシアム」を結成することができた。このコンソーシアムの目的は、新潟大学医歯学総合病院輸血･再生医療部門に設立されたCPCの現場を通じて、将来的な需要が見込まれる細胞培養作業者の育成、教育プログラムコースの新設を目指すことや、簡易無菌キャビネットなど新たな再生医療関連機器の開発を目指すことを目的として、3年の契約で締結され、輸血・再生医療部門の活動を資金面で支えてもらえることとなった。このおかげで、培養作業者を1名追加採用できることとなったのである。

その後、CPCの活動が評価され、培養作業者1名の増員が認められ、また、施設や機器類のバリデーション費用に関しても病院で負担してもらえることとなった。

(3) 情報の収集と文書の整備

細胞プロセッシング室が完成し、培養作業者を雇うことができてほっとしたのも束の間、今度は培養作業者の細胞培養に関する研修、教育をどうするかということや、どうやって施設を管理、運営していくかという問題に直面した。その頃の我々はGMPとは何かということを十分理解していなかったのである。培養作業者の教育については、幸い協力を申し出てくれる再生医療ベンチャー企業があったことから、培養作業者を派遣して研修させることができた。また、GMPについて熟知されている某大学の先生のところへ我々が出張し、CPCに関する管理手順書や作業手順書などの文書体系をどのように作成していくべきかを教えて頂いた。しかし、実際にそれぞれの文書をどう体系づけて作成するか、患者さんに投与する再生医療用細胞や組織を、どうやって安全性を確保しながら作成するかなど見当がつかなかった。

しかし、2007年6月に某再生医療ベンチャーで長年経験を積んだ方を紹介して頂いてメンバーに加わってもらってから、ようやく具体的な方向性を見いだすことができ、プロジェクトのうち、培養骨膜と培養赤芽球についての試験培養を開始することができた。この試験培養のスタートにより、本培養を行うまでの培養手技やそれに伴う作業手順書の作成、試験培養の各段階で行う品質保証に関する試験法やその手順書、環境測定やそれを行う際の手順書などを作成することができた。また、この経験を通じて製品標準書を作成する重要さも痛感した。結局、培養骨膜シート7例、培養赤芽球5例の試験培養を行い、前者については培養各段階における病理組織像、細菌試験、マイコプラズマ試験、グルコース消費試験などの結果を、後者については細胞形態像、表面形質、細菌試験、マイコプラズマ試験、FBS残留試験、生細胞率などの結果とそれぞれの製品標準書、標準作業手順書などの文書を添えて品質管理委員会に提出し、2007年11月に本培養の承認を得ることができた。また、最後に残った培養口腔粘膜についても、その後試験培養を重ね、2008年10月に本培養の承認を得ることができたが、結局3プロジェクトを選択してからすべてを開始できるのに足かけ3年を要したことになる。

Jpn J Ophthalmol
DOI 10.1007/s10384-012-0174-z

CLINICAL INVESTIGATION

Broad-range real-time PCR assay for detection of bacterial DNA in ocular samples from infectious endophthalmitis

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Received: 18 January 2012 / Accepted: 5 July 2012
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Abstract
Background To evaluate a broad-range real-time polymerase chain reaction (PCR) targeting the bacterial 16S rRNA gene for detection of bacterial DNA in infectious endophthalmitis.
Methods The bacterial 16S rRNA gene was measured by quantitative real-time PCR. For the assay, bacterial DNA was prepared from 12 Gram-positive and 4 Gram-negative strains. To determine the optimum method for DNA extraction, four extraction procedures were selected by using DNA extraction program cards with and without the use of lysozyme. To evaluate PCR sensitivity, PCR fragments were amplified from *Staphylococcus aureus* and *Escherichia coli* DNA.
Results DNA extraction using the Bacteria card® without enzymes resulted in detection of all the tested strains at concentrations $\geq 10^7$ copies/mL. Extraction with the Bacteria card® with lysozyme resulted in detection of all the tested strains at concentrations $\geq 10^6$ copies/mL, indicative of no significant difference between the two procedures. DNA extraction using the Virus card®, both with and without enzymes, resulted in reduced efficiency of detection of all strains compared with use of the Bacteria card®. The PCR could detect as few as 1–10 colony-forming units (CFU) in diluted vitreous samples per reaction, and all tested bacterial species known to cause endophthalmitis were detected.
Conclusions Bacterial 16S-specific PCR can comprehensively detect the main causative bacteria of clinically suspected endophthalmitis.

Keywords Endophthalmitis · Bacteria · Polymerase chain reaction

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Introduction

Infectious bacterial endophthalmitis can result both from exogenous infections, for example exposure to infectious agents, trauma, and intraocular surgery, and endogenous infections, for example systemic infectious disorders. It is often difficult to differentiate between inflammation in ocular inflammatory disorders, for example infectious endophthalmitis caused by non-infectious and infectious agents. The standard for diagnosis of invasive bacterial infections used to be microscopic examination and conventional bacterial culture. Although microscopic examination is rapid, the smear test requires a relatively large concentration of bacteria, $\geq 10^4$ colony-forming units (CFU)/mL, to give a positive result [1]. Moreover, identification based solely on morphology is often not possible. Bacterial cultures are often used for differential diagnosis,

Published online: 31 August 2012

but there are several disadvantages, for example cultivation time (24–72 h) and low sensitivity. Inappropriate treatment because of misdiagnosis of infectious endophthalmitis can result in severe tissue damage and vision loss. Because of the difficulty of making proper diagnoses on the basis of the small amounts of ocular samples available, there is a need to consider the collection and preservation of clinical samples, including bacterial DNA, available for diagnostic use. Moreover, some cases involve rapid progression of the ocular infectious disease; therefore, accurate, rapid and comprehensive diagnosis is of great importance.

Polymerase chain reaction (PCR) is used for detection of bacteria in suspected intraocular infections [2–4]. Bacterial PCR is a diagnostic tool that can be used for detection in intraocular specimens, and can be used as an alternative tool for subsequent examination of specimens found to be bacteriologically negative by use of conventional methods, for example cultures and smear tests. Several studies report the presence of the bacterial ribosomal RNA gene (16S rRNA gene) in ocular fluid from patients with infectious endophthalmitis [2–4]. This broad-range PCR can detect a variety of bacterial DNA by use of primers for conserved regions [5, 6], and the combination of broad-range PCR and quantitative PCR for infectious bacterial endophthalmitis is now available [4]. Real-time PCR enables quantification of bacterial loads in a sample. However, the efficiency of extraction of bacterial DNA from ocular fluid by use of a robotic extraction machine is not yet established. Therefore, establishment of a precise extraction procedure is needed for diagnostic clinical use. In addition, broad-range real-time PCR assays are rarely designed to identify bacterial DNA in clinical samples and are not widely used for ophthalmologic diagnosis.

The objectives of this study using broad-range real-time PCR assays were:

1. to determine optimum methods of DNA extraction;
2. to evaluate the sensitivity of the real-time PCR assay in vitreous samples; and
3. to include and test several main causative agents of infectious bacterial endophthalmitis.

Methods

This study was performed in accordance with the tenets of the Declaration of Helsinki and approved by the Institutional Ethics Committees of Tokyo Medical and Dental University.

Bacterial strains

Reference bacterial strains were provided by the National Institute of Technology and Evaluation (NITE, Tokyo, Japan), the NITE Biological Resource Center (NBRC, Chiba, Japan), the Research Institute for Microbial Diseases (RIMD; Osaka University, Osaka, Japan), and the Japan Collection of Microorganisms (JCM, Saitama, Japan). Frequently reported pathogenic bacteria of endophthalmitis were tested, including 12 Gram-positive strains: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus sanguinis*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Corynebacterium diphtheriae*, *Bacillus cereus*, *Clostridium perfringens*, *Propionibacterium acnes*, and *Nocardia asteroides* and 4 Gram-negative strains: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Moraxella lacunata* [7–14].

Before PCR assay, *S. aureus* and *S. epidermidis* strains were cultured in Trypticase soy broth (Difco; BD Diagnostic Systems, Sparks, MD, USA). *S. pyogenes* and *S. sanguinis* strains were cultured in Todd Hewitt broth (Difco) containing 2 % yeast extract (Difco). *E. coli* and *B. cereus* strains were cultured in LB broth (Nacalai Tesque, Kyoto, Japan). The *K. pneumoniae* strain was cultured in nutrient broth (Difco). All bacterial strains were grown until the mid-log phase at 37 °C. Bacterial cells were washed twice with PBS, and then re-suspended in PBS at appropriate concentrations. The remaining strains were dissolved in physiological salt solution without culture.

DNA extraction

DNA extraction was performed using a DNA extraction card (Qiagen EZ1 Advanced card; Bacteria card® or Virus card®; Qiagen, Valencia, CA, USA) and a DNA Kit (Qiagen DNA tissue kit or Qiagen Virus Mini kit; Qiagen) installed on a robotic workstation set for automated purification of nucleic acids (BioRobot E21, Qiagen). Four extraction procedures were used, as follows:

DNA extraction procedure I — Sample preparation: bacterial culture 180 μl + nuclease-free water 20 μl, and extraction method: Bacteria card® + DNA tissue kit

DNA extraction procedure II — Sample preparation: bacterial culture 180 μl + lysozyme 20 μl (50 mg/ml, Nacalai Tesque), and extraction method: Bacteria card® + DNA tissue kit. Bacterial cultures were pretreated with lysozyme

	and incubated for 30 min at 37 °C
DNA extraction procedure III	Sample preparation: bacterial culture 180 μl + nuclease free water 20 μl, and extraction method: Virus card® + Virus Mini kit
DNA extraction procedure IV	Sample preparation: bacterial culture 180 μl + lysozyme 20 μl (50 mg/ml; incubation for 30 min at 37 °C), and extraction method: Virus card® + Virus Mini kit

After DNA extraction, the DNA concentration was measured by use of the Nano drop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), using between 1 and 10 ng/mL bacterial DNA.

Real-time PCR

The primer pairs and TaqMan probe for conserved bacterial 16S rRNA genes and PCR conditions were as described elsewhere [5]. The sense primer (Bac349F) was 5′-AGG CAGCAGTDRGGAAT-3′, the antisense primer (Bac 806R) was 5′-GGACTACYVGGGTATCTAAT-3′, and the TaqMan probe was 5′-FAM-TGCCAG CAGCCGCGG TAATACRDAG-TAMRA-3′. The products were subjected to 45 cycles of PCR amplification (<500 bp), with cycling conditions set at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The real-time PCR was performed using Amplitaq Gold (Applied Biosystems, Foster City, CA, USA) and the Light Cycler 480 II system (Roche, Rotkreuz, Switzerland). Data analysis was performed by using the program of absolute quantification by the Second Derivative Maximum Method installed in Light Cycler 480 II. Standard curves were constructed from serial tenfold dilutions of linearized plasmid DNA as in our previous report [4].

Sensitivity of real-time PCR assay

After informed consent had been obtained, vitreous fluid was collected from 11 patients who received vitreous surgery for non-infectious eye diseases, for example rhegmatogenous retinal detachment, macular edema by branch retinal vein occlusion, and proliferative diabetic retinopathy,. The vitreous samples were diluted threefold with saline before use as a bacterial dilution. The vitreous fluids were centrifuged at 20,000×*g* for 10 min, then the cell pellets were removed.

To evaluate the sensitivity of the real-time PCR assay, bacterial cell numbers were determined by optical density measurements at 600 nm (OD_{600}) in the mid-log phase, and serial dilutions of bacterial culture were plated on the appropriate agar plates, then colony numbers were determined on agar plates. For example, the cell number of *E. coli* at $OD_{600} = 1.0$ was determined to be 8×10^8 CFU/mL, and the cell number of *S. aureus* at $OD_{600} = 1.0$ was determined to be 4×10^8 CFU/mL.

200 μl of a tenfold dilution series from 2.5×10^7 CFU/mL to 2.5×10^1 CFU/mL of *S. aureus* and *E. coli* bacterial culture were centrifuged at 20,000×*g* for 10 min, and pelleted bacteria samples were re-suspended in the same amount of diluted vitreous samples. The bacterial DNA was extracted from 50 μl, from 200 μl of diluted vitreous sample and bacterial pellet, and 10 μl (equivalent to 10^6 CFU/PCR tube to 10^0 CFU/PCR tube) of 50 μl bacterial DNA was used in PCR reactions. The diluted vitreous samples without bacterial cells were used as a negative control.

Results

Analytical sensitivity of broad-range real-time PCR in relation to the four DNA extraction procedures

Four DNA extraction procedures (I–IV) were compared and analyzed. As described in Table 1, the analytical sensitivity of the broad-range real-time PCR was assessed by use of seven representative bacterial strains including five Gram-positive strains (*S. aureus, S. epidermidis, S. pyogenes, S. sanguinis,* and *B. cereus*) and two Gram-negative strains (*E. coli* and *K. pneumoniae*). For negative control samples levels were undetectable for all extraction methods. DNA extraction using Bacteria card® without enzymes resulted in the detection at concentrations of $\leq 10^7$ copies/mL for all strains. Extraction with the Bacteria card® with lysozyme detected concentrations of $\leq 10^6$ copies/mL, indicating there was no significant difference between the two procedures (Table 1). In contrast, DNA extraction with the Virus card® both with and without enzymes resulted in the detection of 10^4–10^8 copies/mL for all strains, which was much less than the detection obtained with the Bacteria card®. Therefore, procedures using Bacteria card® could be used to treat samples of clinically suspected infectious endophthalmitis. In addition, lysozyme treatment is not needed even for detection of Gram-positive bacteria in ocular samples.

Sensitivity of the PCR assay for vitreous samples

PCR results for the prepared vitreous samples showed sensitivity of detection was highest for *S. aureus* bacterial

Table 1 Summary of the analytical sensitivity of broad-range real-time PCR assays in relation to DNA extraction methods

Strain	Number of bacteria DNA (copies/mL)			
	Procedure I	Procedure II	Procedure III	Procedure IV
Gram-positive strains				
Staphylococcus aureus	10^8	10^7	10^5	10^7
Staphylococcus epidermidis	10^8	10^8	10^8	10^8
Streptococcus pyogenes	10^7	10^8	10^4	10^7
Streptococcus sanguinis	10^7	10^8	10^6	10^8
Bacillus cereus	10^8	10^8	10^7	10^7
Gram-negative strains				
Escherichia coli	10^7	10^6	10^7	10^5
Klebsiella pneumoniae	10^7	10^7	10^5	10^6
Negative control	<10	<10	<10	<10

DNA extraction procedure I: sample: bacterial culture 180 µl + nuclease-free water 20 µl, and extraction method: Bacteria card® + DNA tissue kit. procedure II: sample: bacterial culture 180 µl + lysozyme 20 µl, and extraction method: Bacteria card® + DNA tissue kit. procedure III: Sample: bacterial culture 180 µl + nuclease free water 20 µl, and extraction method: Virus card® + Virus Mini kit. procedure IV: sample: bacterial culture 180 µl + lysozyme 20 µl, and extraction method: Virus card® + Virus Mini kit

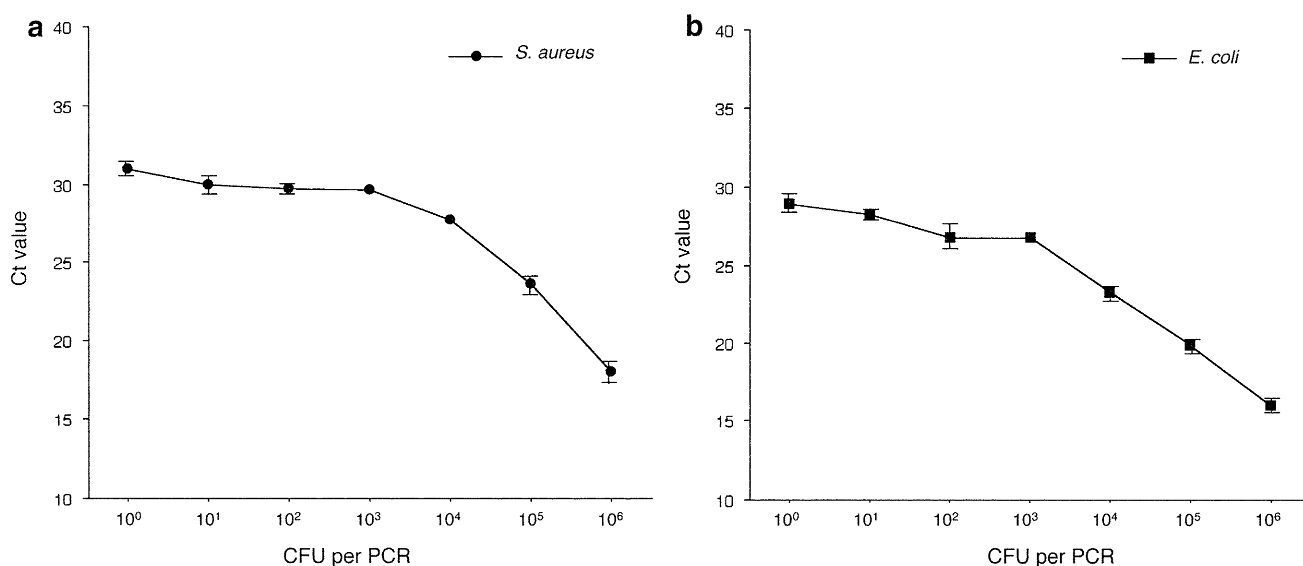

Fig. 1 Analytical detection ranges and sensitivities of a broad-range real-time PCR assay in diluted vitreous samples. **a** Detection of *S. aureus*. **b** Detection of *E. coli*. Results shown are the means and standard deviations of three independent experiments

DNA (concentration $\geq 10^1$ CFU per PCR; Fig. 1a). There was no detection in the negative controls of nuclease-free water. PCR of vitreous sample mixed with *E. coli* resulted in C_t values similar to those for *S. aureus*, i.e., concentration of 10^0 per PCR (Fig. 1b), and there was no detection in the negative controls.

Detection of bacterial DNA of the main causative agents of infectious endophthalmitis by broad-range real-time PCR

For the assay, bacterial DNA was extracted from 200 µl bacterial culture using DNA extraction procedure I. Use of DNA extraction with the Bacteria card® without enzymes and broad-range real-time PCR assay resulted in the detection of concentrations between 5.8×10^3 and 3.5×10^5 copies/mL for all 16 strains. There was no detection in the negative controls of nuclease-free water. Results are shown in Table 2.

Discussion

In this study, we evaluated a broad-range real-time PCR targeting bacterial 16S rRNA genes for detection of bacterial DNA in ocular samples of infectious endophthalmitis.

Table 2 Broad-range real-time PCR detection of bacterial DNA in main causative agents of infectious endophthalmitis

Strain	Clone no.	DNA (ng/mL)	C_t value	Copies/mL
Gram-positive strains				
Staphylococcus aureus	NBRC12732	7.3	28.7	1.3×10^4
MRSA	JCM8702	7.0	29.1	1.0×10^4
Staphylococcus epidermidis	JCM2414	6.0	27.9	1.7×10^4
Streptococcus pyogenes	RIMD 3123004	7.2	28.0	1.6×10^4
Streptococcus sanguinis	JCM5708	3.6	29.1	9.7×10^3
Streptococcus pneumoniae	NBRC102642	8.2	25.7	9.4×10^4
Enterococcus faecalis	JCM20313	2.0	24.0	1.1×10^5
Corynebacterium diphtheriae	JCM1310	4.4	25.2	6.1×10^4
Bacillus cereus	JCM20266	4.9	26.8	2.9×10^4
Clostridium perfringens	JCM1290	6.1	29.9	5.8×10^3
Propionibacterium acnes	JCM6425	1.4	28.3	1.5×10^4
Nocardia asteroides	NBRC14403	8.0	28.7	1.3×10^4
Gram-negative strains				
Escherichia coli	JCM20135	8.7	23.2	1.5×10^5
Klebsiella pneumoniae	JCM1662	7.5	26.8	2.9×10^4
Pseudomonas aeruginosa	JCM6425	5.6	23.7	3.5×10^5
Moraxella lacunata	JCM20914	3.2	25.8	8.9×10^4

Levels were undetectable in the negative control sample (<10 copies/mL) on PCR assay

MRSA methicillin-resistant *Staphylococcus aureus*

Using this broad-range PCR, we are able to measure amplification of the bacteria 16S target ribosomal RNA genes. To detect different bacterial species, we choose the PCR primers and probe which were constructed within the conserved region of bacterial 16s ribosomal RNA. We evaluated four DNA extraction procedures used for broad-range real-time PCR assays in the detection of bacterial DNA. The broad-range real-time PCR described herein detected as few as 1–10 CFU in diluted vitreous per reaction. In addition, the bacterial 16S-specific broad-range real-time PCR assay could detect the presence of 16 causative bacterial species of infectious endophthalmitis. Thus, the broad-range real-time PCR could comprehensively detect the main causative bacteria in suspected infectious endophthalmitis cases.

The appropriate DNA extraction procedure for verification of bacterial infection by PCR is still controversial. Most studies of broad-range real-time PCR for bacterial infection detection have reported use of commercial kits, enzyme treatment, freezing and thawing or boiling, mechanical disruption, or a combination of these methods [6, 15–17]. In general, pretreatment using bactericidal enzyme is needed for bacterial cell-wall destruction, and several investigators report the presence of lysozyme resistance in Gram-negative bacteria species such as *E. coli* [18] and *P. aeruginosa* [18], and Gram-positive bacteria such as *S. pneumonia* [19]. However, this study found no significant difference between use of the Bacteria card® procedure and the Bacteria card® plus lysozyme-pretreatment procedure for extraction of DNA from the samples. The reasons for this are not clear, but it is assumed it may depend on:

1. the kind of enzyme used; and
2. which bacteria species are treated.

Thus, a combination of several enzyme treatments should be tried whenever possible.

Diagnosis of ocular infectious diseases, including bacterial endophthalmitis and other forms of ocular inflammatory diseases, is often difficult because of the difficulty in obtaining results from the small amounts of ocular samples, extracted from aqueous humor and vitreous fluids, available. There are insufficient amounts of the samples to enable PCR testing and additional examination to determine whether the infectious antigens causing the ocular inflammatory diseases are from a bacterial, viral, fungal, or parasitic infection.

In this study, we conducted various DNA extraction procedures to determine the best DNA extraction method. Compared with the use of the Virus card®, DNA extraction using the Bacteria card® had higher detection efficiency for all the representative strains tested. DNA extraction performed with the Virus card® detected bacterial DNA, but was not as efficient for strains that have a thick cell wall and a capsule, for example *S. aureus, S. pyogenes*, and *K. pneumoniae*. Thus, DNA extraction with the Bacteria card® should be considered for detection of clinically suspected intraocular bacterial infection.

The minimum detection limits of our broad-range real-time PCR assay after DNA extraction using the Bacteria

card® without enzymes was between 10^0 and 10^6 CFU per PCR for the bacterial species investigated. The PCR results from the prepared vitreous sample had the best sensitivity for detection of selected bacterial DNA, for example *S. aureus* and *E. coli* at concentrations of $\geq 10^0$–10^1 CFU per PCR. Zucol et al. [6] report that the sensitivity of their broad-range real-time PCR assay targeting the bacterial 16S rRNA gene was a concentration of $\geq 10^3$ CFU per PCR for detection of *S. aureus* and a concentration of $\geq 10^2$ CFU per PCR for detection of *E. coli*. In addition, the minimum detection limits for *S. aureus* and *E. coli* were determined to be in the range 10–10^3 CFU or CFU equivalents per PCR. Thus, the minimum detection limit of our PCR assay is among the lowest reported so far for these two bacterial species.

In a previous report by Vollmer et al. [20], serum and urine samples were shown to have at least an equal effect on the Ct values, whereas blood and tracheal secretion samples had stronger effects. They suggest that the delayed C_t values of blood sample (EDTA-anti-coagulated) are mainly affected by background human DNA, whereas the viscous character of samples primarily affected the C_t values of tracheal secretion samples. Because our vitreous samples included less human DNA and the diluted sample was actually non-viscous, detection of bacterial DNA from prepared vitreous samples was shown to be highly sensitive.

Recently, we reported that broad-range real-time PCR of the bacterial 16S rRNA gene is a useful tool for clinically diagnosing suspected bacterial endophthalmitis [4]. In an earlier clinical study, we successfully detected bacterial 16S DNA in all cases of bacterial endophthalmitis ($n = 18$), with the exception of one patient. The single PCR-negative patient was suspected of having infectious endophthalmitis but had no bacteria in his ocular sample; *K. pneumoniae* was detected by biopsy culture of liver infection. However, as described in this study, our bacterial 16S-specific broad-range real-time PCR can detect candidate bacterial DNA including *K. pneumoniae* (Table 2). *K. pneumoniae* is a common cause of endogenous infectious endophthalmitis, a disease that frequently results in poor vision. *K. pneumoniae* endophthalmitis is strongly associated with the presence of liver abscesses and an underlying diabetic condition. We collected aqueous humor samples from the patient after informed consent had been obtained. Had a vitreous sample also been obtained, we might have been able to detect the bacterial DNA, because *K. pneumoniae*-associated endophthalmitis often results from hematogenous dissemination. To make an accurate diagnosis, sample preparation of clinical specimens is very important. A vitreous or retinal biopsy sample should be collected in such cases because inflammation often occurs in the subretinal area around the choroid because of endogenous infections.

In conclusion, we were able to use a broad-range real-time PCR method to measure the amplification of target ribosomal RNA genes, for example the bacterial 16S rRNA gene, indicating the suitability of this assay for screening for increased levels of bacterial genes in samples. Importantly, these PCR assays may be used for detection of candidate bacterial species that cause infectious endophthalmitis. The detection limit of our real-time PCR assay is one 16S rRNA gene copy per PCR. Thus, this PCR assay enables rapid screening for bacterial infection in a variety of clinical specimens from the eye.

Acknowledgments The authors thank Ikuyo Yamamoto, Shizu Inoue, and Chizuru Kato for providing technical assistance and the doctors of the Uveitis Group at Tokyo Medical and Dental University Hospital for sample collection. This work was supported by the Comprehensive Research on Disability, Health and Welfare, Health and Labor Sciences Research Grants, Ministry of Health, Labor and Welfare, Japan.

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Virological Analysis in Patients with Human Herpes Virus 6–Associated Ocular Inflammatory Disorders

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

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

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

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

Human herpesvirus 6 (HHV-6) is the causative agent of 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

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Supported by Comprehensive Research on Disability, Health and Welfare, Health and Labour Sciences Research Grants, Ministry of Health, Labour, and Welfare, Japan.

Submitted for publication April 26, 2012; revised May 29, 2012; accepted June 10, 2012.

Disclosure: **S. Sugita**, None; **N. Shimizu**, None; **K. Watanabe**, None; **M. Ogawa**, None; **K. Maruyama**, None; **N. Usui**, None; **M. Mochizuki**, None

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Investigative Ophthalmology & Visual Science, July 2012, Vol. 53, No. 8
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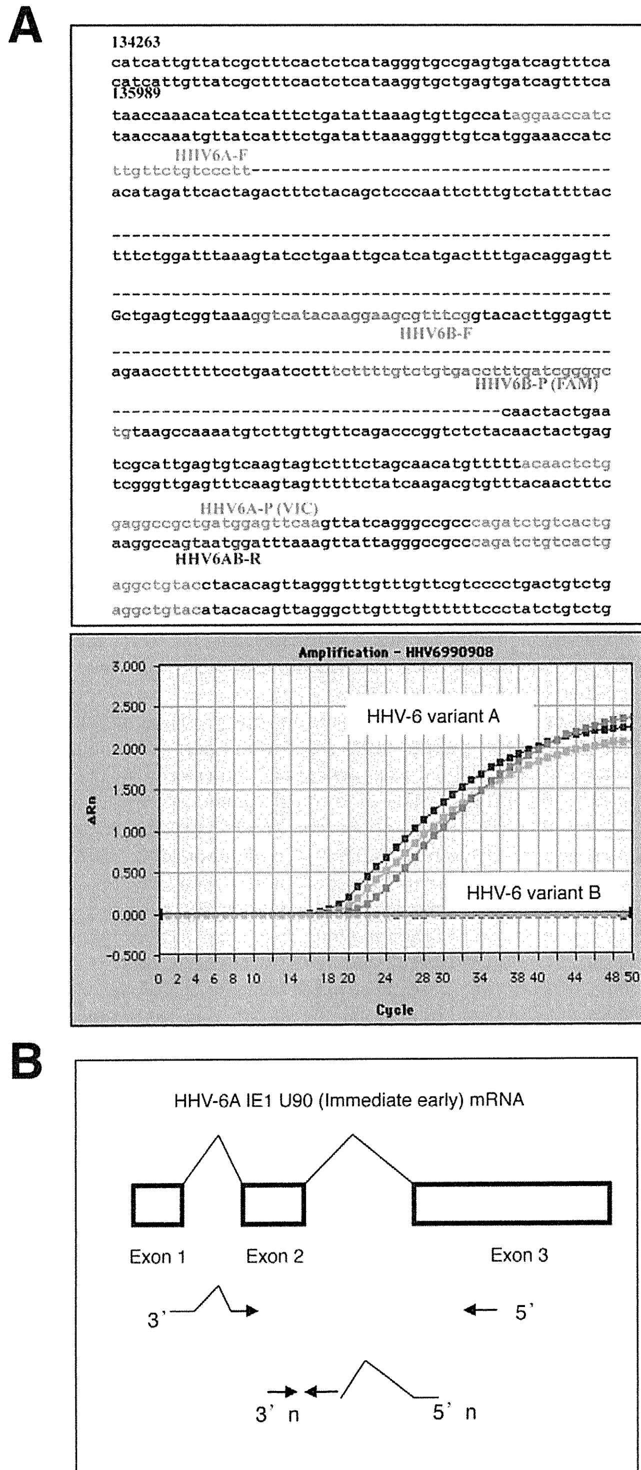

Figure 1. Amplification of HHV-6–specific DNA and mRNA. **(A)** TaqMan probes and primers used to amplify HHV-6 DNA (HHV-6A and HHV-6B). HHV-6 subtypes were identified using PCR with variant-specific primers and probes (*lower graph*). **(B)** Nested RT-PCR primers were designed to amplify HHV-6A mRNA.

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 endothelitis	0.5	33	Edema	Cell 2+	Mutton fat	None	None	PCR (-)	HSV-1 corneal endothelitis
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

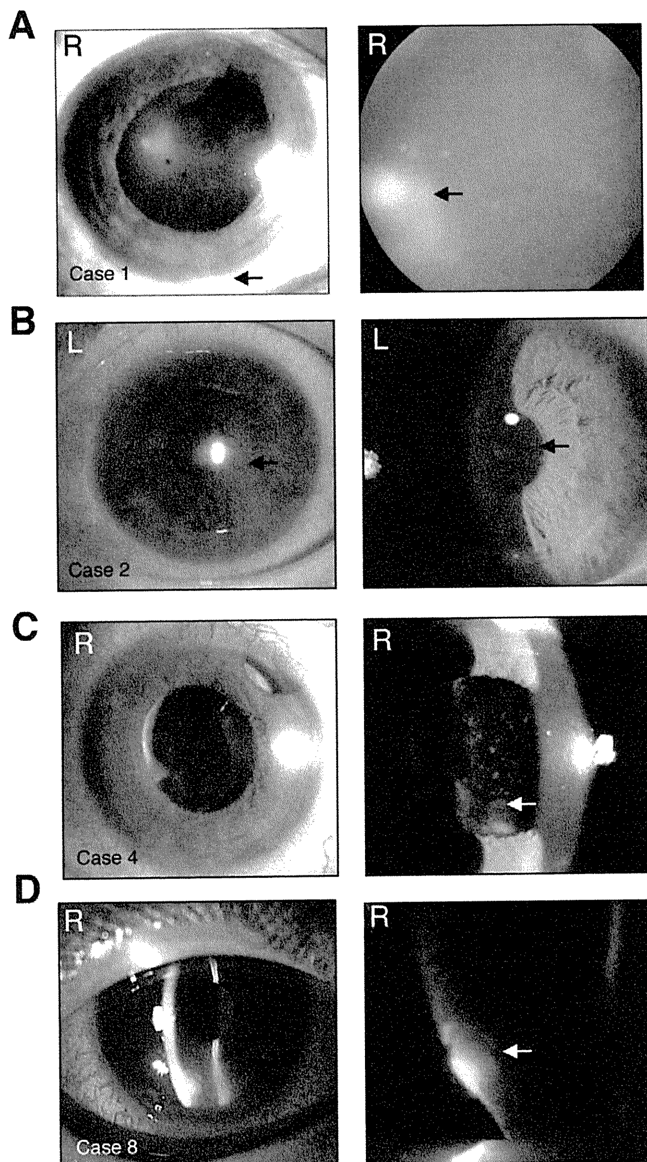

FIGURE 2. Slit-lamp and fundus photographs for HHV-6 infections. **(A)** Case 1: A case of ocular toxocariasis. Slit-lamp examination of right eye (RE) disclosed ciliary injection, moderate mutton-fat keratic precipitates (KPs), and severe anterior chamber cells with hypopyon (*arrow*). Funduscopic examination 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