[表1] 感染性眼内炎と診断された7症例の眼内液を用いた細菌 broad-range PCR結果のまとめ

症例	最終診断名	細菌16S rRNA領域 定量PCR	培養結果	塗抹結果
1	感染性眼内炎	1.3×10^7 copies/ml	陽性(*Psuedomonas* ssp.)	グラム陰性桿菌
2	感染性眼内炎	1.5×10^8 copies/ml	陰性	陰性
3	感染性眼内炎	1.4×10^6 copies/ml	陽性(*Enterococcus*)	グラム陽性球菌
4	感染性眼内炎	1.1×10^4 copies/ml	陰性	グラム陽性球菌
5	術後感染性眼内炎	2.8×10^8 copies/ml	陽性(*Staphylococcus*)	陰性
6	術後遅発性眼内炎	8.1×10^7 copies/ml	陰性	陰性
7	術後遅発性眼内炎	1.7×10^3 copies/ml	陰性	陰性

臨床診断で眼内炎と診断された7症例の硝子体または前房水を用いて細菌 broad-range PCR を施行した．同検体の培養と塗抹検査も施行した．

遺伝子のPCRによる増幅は，25Fプライマーを用いて16S rDNAの前半約500bpを解析する．その増幅したPCR産物をダイレクトシークエンスする．シークエンシングにはABIアナライザーを用いて配列を解析し，その後genebank blastで一致性を検索する．100%一致(あるいは98%以上)する菌を同定菌としている．われわれの施設では，真菌の中で眼科関連性が高いとされるカンジダとアスペルギルスの18S rRNA遺伝子領域のbroad-range PCRも行っている．

■感染性眼内炎眼内液を用いた細菌broad-range定量PCRの有用性の検討

感染性眼内炎の診断には一般的には眼内液の培養，塗抹標本などが行われるが，われわれは感染性眼内炎患者の眼内液検体を用いて細菌broad-range定量PCR法を施行し，それらについて解析した．対象は，眼内手術や眼外傷後の感染性眼内炎と臨床診断された7眼の感染性眼内炎患者で，それらの眼内液(硝子体液または前房水)からDNA抽出後，細菌broad-range定量PCRを行った．また同検体を同時に培養，塗抹標本(スメア)検査も行った．

結果のまとめは**表1**に示す．感染性眼内炎の7眼すべて(100%)で高コピー数の細菌DNAが検出された($1.7 \times 10^3 \sim 2.8 \times 10^8$ copies/ml)．この感染性眼内炎の7眼で培養陽性は3例で，陰性は4例であった．同様に，塗抹標本でも3例で陽性，4例で陰性であった(表1)．また，この感染性眼内炎7眼中6眼は抗菌薬の全身投与と局所投与により軽快した．これらの結果より，感染性眼内炎の眼内液を用いた診断で培養や塗抹検査では陰性になることもあり，この細菌16S broad-range定量PCR法は有用と考えられる．Chiquetらによると，術後感染性眼内炎患者の眼内液検体の多くは早期から抗生物質が投与されており培養検査では陰性になることが多く，しかし菌DNAは残存していることからbroad-range PCRは有用であると報告している[2]．次の項でこのbroad-range PCRが有用であった症例を紹介する．

■broad-range PCRが有用であった代表症例

症例1

42歳，女性，術後眼内炎疑いで紹介．右眼PEA＋IOL施行，その後急激な視力低下を自覚した．右眼光覚弁，前房内フィブリンと前房蓄膿を伴う眼内炎症がみられた(**図2**)．硝子体を用いた検査では，培養で*S. aureus*以外の*Staphylococcus*が検出されたが，塗抹は陰性だった．細菌16S rRNA領域broad-range定量PCRで，細菌DNAが2.8×10^8 copies/mlと陽性を示していた．最終診断は術後細菌性眼内炎とし，硝子体手術(硝子体切除＋IOL除去＋前房洗浄＋術中抗生物質還流)に術後抗生物質全身投与で軽快した．

症例2

53歳，男性，芝刈り後の眼内炎疑いで紹介，受診．右眼手動弁，前房蓄膿の激しい眼内炎症がみられた

(図3). 硝子体を用いた検査では，培養で *Enterococcus* 属が検出され，塗抹でグラム陽性球菌が同定された．同 broad-range PCR で，細菌 DNA が 1.4 × 10^6 copies/m*l* と高コピー数を示していた．手術時の所見や画像上では眼内異物が発見できなかったが，最終診断は外傷性感染性眼内炎とした．その後のブラスト解析にて *Enterococcus faecalis* が同定された．

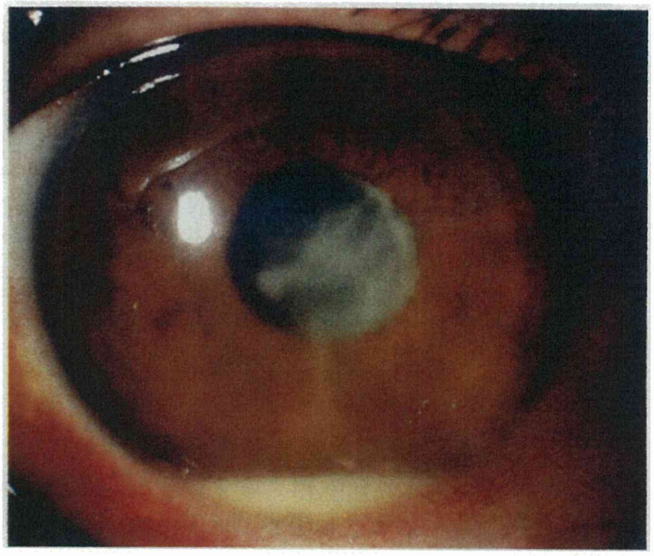
■broad-range PCR検査システムの今後の検討課題

この新しい PCR 検査システムは，細菌は 16S rRNA 領域，真菌は 18S rRNA 領域を増幅させることができ臨床の場では多いに役に立つことが推定される．これらは何の菌までかはわからなくてもこれらの感染を否定することができ，治療の中心がステロイドのぶどう膜炎分野ではこれらの PCR が広く使用されるようになってきている．また将来的にはブラスト解析の組み合わせで培養よりも早期に菌の同定まで行えるようになるであろう．今後の課題は，症例数を増やして有効性を検討する必要があること，また PCR で陽性であった場合，コンタミネーションと病因抗原との判別が重要となる．加えて他の検査結果との一致性，例えば細菌培養で検出された菌と PCR で同定された菌との一致性，あるいは臨床所見との一致性や治療効果との一致性など検討する課題は少なくない．

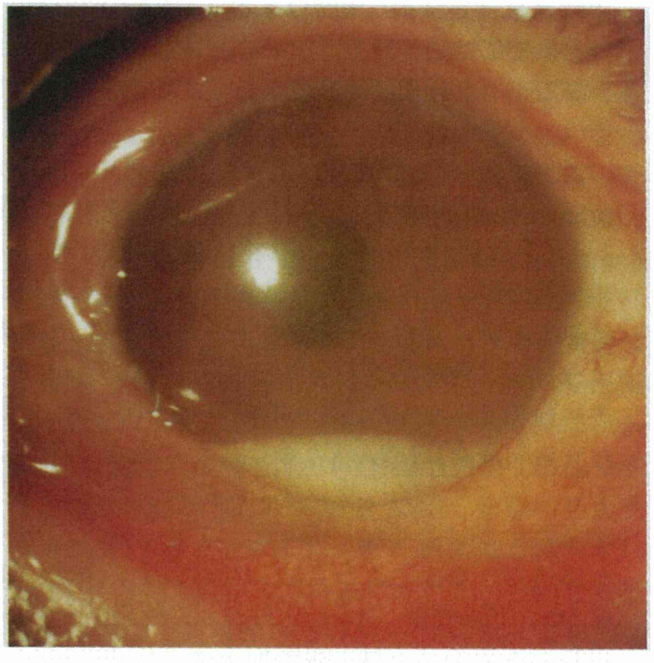
文献

1) Takai, K et al : Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Appl Environ Microbiol 66 : 5066-5072, 2000

2) Chiquet, C et al, French Institutional Endophthalmitis Study Group : Eubacterial PCR for bacterial detection and identification in 100 acute postcataract surgery endophthalmitis. Invest Ophthalmol Vis Sci 49 : 1971-1978, 2008



[図2] broad-range PCR が有用であった代表症例1
術後細菌性眼内炎の症例．硝子体を用いた細菌 16S rRNA 領域 broad-range 定量 PCR で，細菌 DNA が 2.8 × 10^8 copies/m*l* と陽性を示した．



[図3] broad-range PCR が有用であった代表症例2
外傷性感染性眼内炎の症例で，同様の broad-range 定量 PCR で 1.4 × 10^6 copies/m*l* と高コピー数を示した．

Advanced Techniques

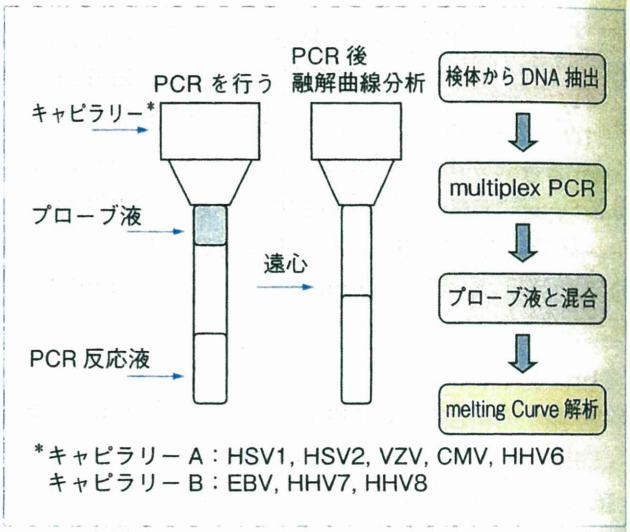
multiplex PCR

東京医科歯科大学大学院医歯学総合研究科視覚応答調節学 **杉田 直**

■multiplex PCR法とは？

polymerase chain reaction（PCR）とはポリメラーゼ連鎖反応のことで，DNAポリメラーゼ反応を利用した微量DNAの増幅方法である．2種類のDNA断片のプライマーを用いてDNAの特定部位を挟みながらDNAを合成するDNAポリメラーゼ酵素を用いてDNA鎖の合成反応を起こさせる．この反応の繰り返しにより，目的のDNA特定部位を約数百万倍程度まで増幅させることが可能である．このDNA合成のプロセスには数分しかかからないことから，このPCRの利用が急速に広まっている．PCR法は遺伝子配列の決定や遺伝子の定量など，遺伝子研究の基本技術として確立されている．臨床の場では，ウイルス，クラミジア，細菌，真菌などの診断方法として応用されている．眼科領域ではぶどう膜炎，眼内リンパ腫には診断目的で，緑内障あるいは網膜色素変性症では原因遺伝子検索が目的で広く応用されている．現在，ぶどう膜炎，特にウイルス感染で確定診断のためにはPCRは有効な検査手段となっている．実際ウイルスDNAのゲル内の検出までには数時間で可能であること，さらにサザンブロットを行えば，感度を上昇させることもできる．

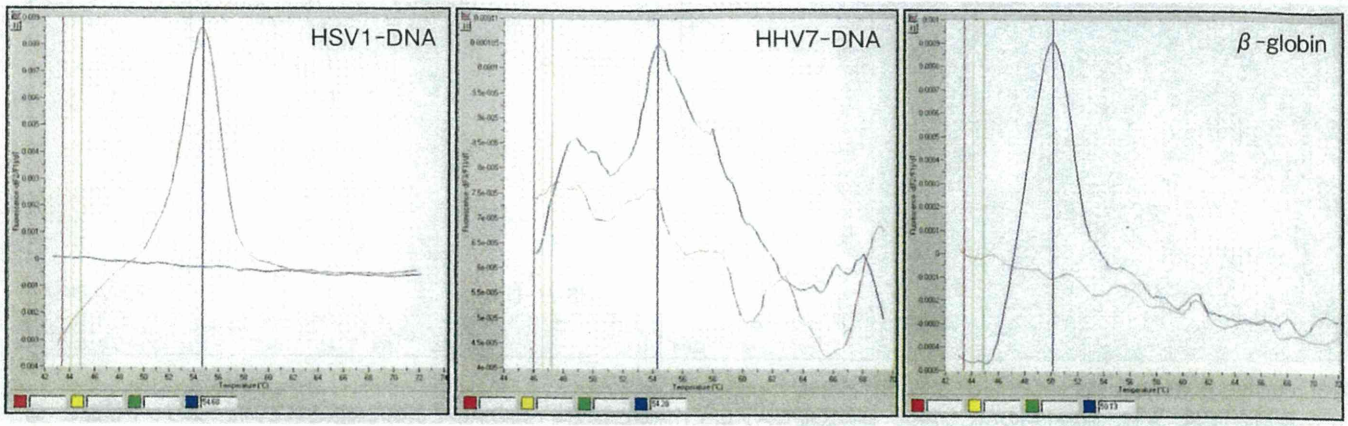
近年，このPCRを応用して開発されたのがmultiplex PCR（多項目迅速PCR）検査である（図1）[1~3]．このmultiplex PCR検査の最大の特徴は，数種類のウイルスなどの外来性抗原を同時に迅速に検出できる．以前の一般的に行われていたPCRのようなゲル内のバンド検出で判定するのではなく，融解曲線で陽性か陰性かの判定を行う（図2）．曲線が大きい場合，DNA量が多いことがわかり半定量できる利点がある．サンプル調整からPCRにかかる所要時間はわずか2時間弱と従来のPCRの中でも迅速で，場合によっては10項目以上の外来性抗原DNAが陽性か，あるいは陰性かの判定ができる．眼科領域での利点は，眼表面炎症性疾患（角膜炎，結膜炎など）の涙液検体は複数の外来性抗原が検出される可能性があり，このmultiplex PCR検査は有用と思われる（図2）．また，眼科検体が多くの症例で微量であるので，この手法は検体量の少ないこの領域ではきわめて有用である．



［図1］multiplex PCR検査法
このmultiplex PCR（多項目迅速PCR）検査は，数種類（多い場合は10種類以上が可）のウイルスなどの外来性抗原を同時に迅速に検出できる新しいPCR検査システムである．眼局所検体からDNAを抽出後，Accuprime Taqを用いてそれぞれのウイルス特異的プライマーを混合して，multiplex PCRを行う．数種類のウイルスを2つのキャピラリーを用いて同時に検査する．PCR反応後，ハイブリダイゼーションプローブの混合液とPCR産物を混合し，melting Curve解析（融解曲線分析）を行い，ウイルスの同定を行う．これらはTm値（melting temperature，融解温度）が重ならないように設定したプローブによってウイルスの種類を判定する．

■方法および利点・欠点

multiplex PCRにより増幅されたDNA断片が目的の遺伝子に特異的であるかを測定するために，そのPCR産物は遺伝子特異的プローブを用いたハイブリダイゼーションによる検出を行う．ほとんどすべてのPCR産物に対して通常のプローブとビオチン標識プローブの両方を用いて証明される．検体より核酸抽出にmultiplex PCRを行うが，Accuprime Taq（インビトロジェン社など）を用いて，それぞれの検出したい目的の抗原特異的プライマーを混合してPCRを行う（図1）．その後，ハイブリダイゼー



[図2] multiplex PCRの結果グラフ
融解曲線カーブで陽性か陰性かの判定を行う．この症例はヘルペス性角膜炎疑い患者の涙液をインフォームドコンセントのもと採取して，患眼涙液からHSV1-DNAが検出された．HSV1-DNAはTm値が約55℃で曲線が検出されるように設定している．加えて，同検体からHHV7-DNAも検出されていた．他のヘルペスウイルスDNA(HSV2, VZV, EBV, CMV, HHV6, HHV8)はすべて陰性で，健眼の涙液はHHV1-HHV8すべて陰性であった．β-globin - 内因性コントロールで，検体からDNAが十分取れていることを示す．

ションプローブの混合液とPCR産物を混合し，melting Curve解析(融解曲線分析)を行う．melting Curve解析とは，実際には95℃でPCR産物を1本鎖にしたところから40℃に温度を下げてプローブとハイブリダイズさせるとFRET(fluorescence resonance energy transfer)により蛍光が発せられる．再び温度を徐々に上昇させながら蛍光強度をモニタリングしていくとFRETにより発する蛍光が上昇温度により各種抗原プローブ固有のTm値に達したところでFRETが解消し消光する．時間に対する蛍光強度グラフを微分して得られた異なる各種抗原プローブのピーク値によって外来抗原種を判定することができる．

このPCRの欠点は，複数のPCR反応が同時に一つの反応系で進むようにするため，プライマーの設定，プライマー混合比の複雑な検討が必要であり，技術と時間を要する．また，陽性の外来性抗原種はTm値によって判定されるため，検出するTm値はハイブリプローブのTm値に依存している．このTm値の算出が複雑で正確な設計には多くの作業が必要となる．

一般的なPCRは通常，特異的な配列を増幅するプライマーを1組しか使用していない．一方，multiplex PCRは同時に多くの配列を増幅するための複数組のプライマーを使用する．最大の利点は，同時に迅速に複数の外来性抗原を網羅した検査システムが確立できる．しかし，1つのチューブ中に多数のPCRプライマーが存在することは，ミスプライミングPCR産物，プライマーダイマー，短いDNA断片に偏った増幅などの多くの問題の原因になる可能性がある．

■multiplex PCR法を用いた検査手順
(具体例—ウイルススクリーニングPCR検査—)

1. 症例

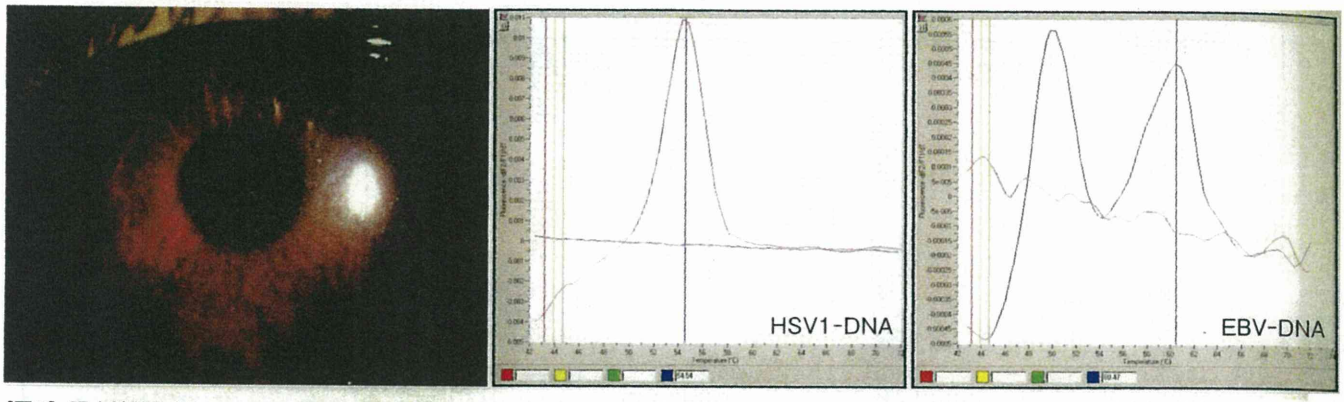
症例は58歳，女性．原因不明の右眼ぶどう膜炎，高眼圧症でステロイド点眼と眼圧降下薬の点眼を長期に点眼されていた．特発性ぶどう膜炎の診断で紹介，受診．スリット検査で，豚脂様角膜後面沈着物がびまん性に付着していた(**図3**)．右眼に高眼圧症と軽度の前房内炎症がみられた．ウイルス感染を疑い，インフォームドコンセントによる同意のもと前房水0.1m*l*を採取した．

2. 核酸抽出

前房水から核酸抽出キット(EZ1 Virus Mini Kit (キアゲン社))および自動核酸抽出装置(BioRobot E21)を用いて核酸を抽出した．

3. multiplex PCR検査

ヘルペスウイルス感染を考え，ヒトヘルペスウイルス属HSV1(HHV1)，HSV2(HHV2)，VZV(HHV3)，EBV(HHV4)，CMV(HHV5)，HHV6，HHV7，HHV8を検討した．multiplex PCRを用いて上記8種類のウイルスを2組に分けて下記の2つのキャピラリーを用いて同時にスクリーニングした(図1)．キャピラリーA：HSV1，HSV2，VZV，HHV6，CMV，キャピラリーB：EBV，HHV7，HHV8．PCR条件



[図3] 眼内検体を用いて multiplex PCR を施行した代表症例
症例は58歳，女性．原因不明のぶどう膜炎，高眼圧症でステロイド点眼と眼圧降下薬の点眼を長期に点眼されていた．スリット検査で，写真のような豚脂様角膜後面沈着物がびまん性に付着していた．初診時に採取した前房水から HSV1-DNA が検出された．同検体から EBV-DNA も同定され，抗ウイルス薬の内服と眼軟膏，ステロイド点眼でやがて消炎した．

は，denature 95℃ 2秒，PCR 95℃ 2秒，58℃ 15秒，72℃ 15秒を40サイクル，extension 40℃ 30秒行った．その後，ハイブリダイゼーションプローブの混合液と PCR 産物を混合し，melting Curve 解析を行い，ウイルスの検出を行った．これらは Tm 値(melting temperature，融解温度)が重ならないように設定したプローブによってウイルスの種類を判定した．なお，HSV の検出に関しては，プライマーは HSV1 と HSV2 の両方の型に共通な部分に設定している．しかし，プローブは HSV2 のゲノム配列とは完全一致しているのに対し，HSV1 のゲノム配列とは2塩基不一致となっている．そのため，HSV2 陽性の場合は HSV1 陽性の場合よりも Tm 値がおよそ15℃低くなり，両者を明確に判別できるようにした．

4. 検査結果

前房水から multiplex PCR 検査で，HSV1-DNA が検出された(図3)．また同検体から EBV-DNA も同定された．その他 HSV2，VZV，CMV，HHV6，HHV7，HHV8-DNA はすべて陰性であった．治療にバラシクロビル内服とデキサメタゾン点眼を用いてやがて消炎した．

multiplex PCR の今後の展望

近年の PCR システムの改良により，多種のウイルスなどの外来性抗原を同時にかつ迅速にスクリーニングして，その後異なったプライマーとプローブの組み合わせでウイルス量の定量化検査(リアルタイム PCR)をする検査システムが報告されている[1~3]．その他の利点として，多くの感染性ぶどう膜炎や眼内炎を否定する目的で使用でき，治療の中心がステロイドのぶどう膜炎分野ではこれらの PCR が広く使用されるようになってきている．理論的にはすべての外来性抗原を PCR で検出することが可能で，今後は眼科関連性のある外来性微生物をすべて網羅できる検査システムの開発が待たれる．

文献

1) Sugita, S et al : Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. Br J Ophthalmol 92 : 928-932, 2008

2) 杉田　直ほか：急性網膜壊死患者眼内液の多項目迅速ウイルス PCR およびリアルタイム PCR 法によるヘルペスウイルス遺伝子同定．日眼会誌 112 : 30-38, 2008

3) Kido, S et al : Association of varicella-zoster virus (VZV) load in the aqueous humor with clinical manifestations of anterior uveitis in herpes zoster ophthalmicus and zoster sine herpete. Br J Ophthalmol 92 : 505-508, 2008

PCR法の利点・欠点（定性PCR, RT-PCR, multiplex PCR, real-time PCR, broad-range PCR）

杉田　直
東京医科歯科大学医学部眼科学教室

PCR法とは

眼科領域では検体量が特に微量なためこのPCR遺伝子検査は欠かせない診断方法となった.

ポリメラーゼ連鎖反応（PCR）法は，DNAポリメラーゼ反応を利用した微量DNAの増幅方法である．2種類のDNA断片，プライマーを用いてDNAの特定部位を挟みながらDNAを合成する酵素（DNAポリメラーゼ）を用いてDNA鎖の合成反応を起こさせる．この反応の繰り返しにより，目的のDNA特定部位を数十万～数百万倍程度まで増幅させることが可能である．このDNA合成のプロセスには数分しかかからないことから，近年このPCR法の利用が急速に広まった．

PCR法は遺伝子配列の決定や遺伝子の定量など，遺伝子研究の基本技術として確立されている．最近では臨床の場で，ウイルス，クラミジア，細菌，結核菌などの診断方法として応用されている．眼科領域では，ぶどう膜炎，眼ウイルス感染症，眼内リンパ腫には診断目的で，緑内障あるいは網膜色素変性症では原因遺伝子検索が目的で広く応用されている．現在，ぶどう膜炎，特にウイルス感染症では迅速な確定診断のためにはPCR法はきわめて有効な検査手段となっている．実際ウイルスDNAのゲル内の検出までには数時間で可能であること（1），さらにSouthernブロット法を行えば，感度を上昇させることもできる．

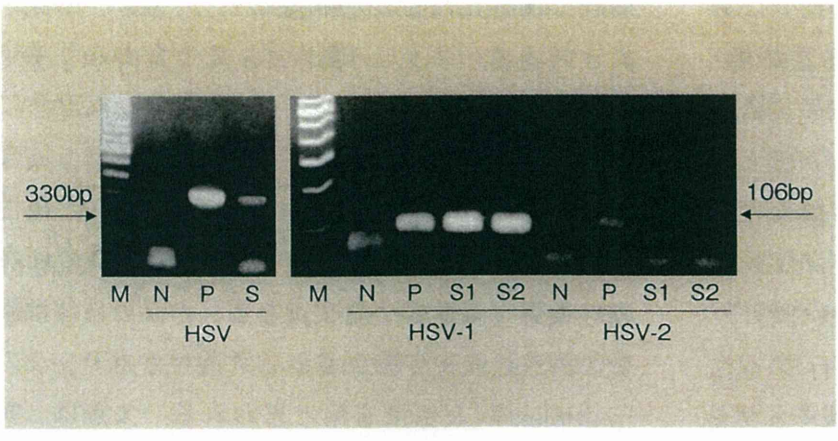
PCR法の種類とその利点・欠点

臨床診断のための検査法としてすでに定着しているPCR法は，基礎実験の領域でも最も一般的な実験手法である.

以下に，現在の代表的なPCR法についてその目的や方法，その利点・欠点などを述べる．

定性PCR法

以前は多くの眼内炎症性疾患で，病因抗原が病巣（眼局所）から検出されずに疑いのまま加療されていた時代があった．ぶどう膜炎の場合，眼局



1 定性PCR写真

PCR検査で診断された単純ヘルペスウイルス1型（HSV-1）感染による角膜ぶどう膜炎の症例．初診時に採取された前房水検体からゲル内の330bpのところにHSV-DNAの陽性バンドが検出されている（左ゲル写真）．右ゲル写真は再発時の前房水検体と初診時の検体を使用してHSV-1とHSV-2を別々に判別できるプライマーを使用してPCRを施行した結果．HSV-1-DNAが初診時および再発時の前房水の両者で陽性（106 bp），HSV-2は陰性．M：100bpマーカー，N：negative control（陰性コントロール），P:positive control（陽性コントロール），S：前房水検体，S1：前房水検体（再発時），S2：前房水検体（初診時）．

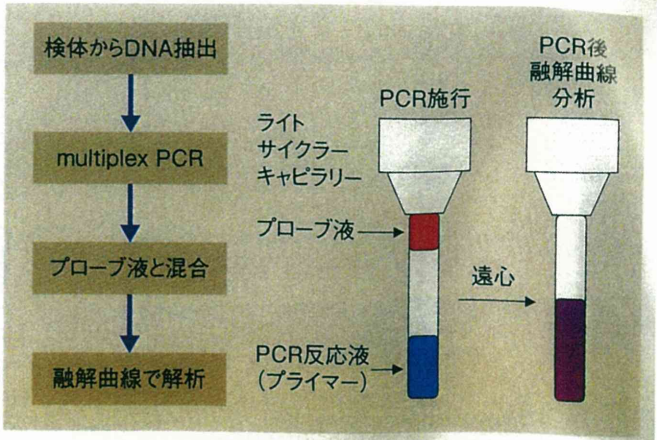
所からウイルスなどの外来性抗原DNAが同定できれば，ウイルス性ぶどう膜炎と診断でき，その病因的価値が高くなる．診断検査目的には通常前房水が用いられるが，得られる検体量は0.1mL程度であることから可能な検査は限られる．硝子体の場合は約0.5～1.0 mLあり，PCR法だけではなく抗体測定，細胞診，培養など多くの検査が可能である．1に示すように，ゲル内に目的のバンドが検出されれば「陽性」と判断される．

PCR法の全般にいえる利点として，微量検体でも検査可能である点があげられる．眼科検体には前房水，硝子体液，涙液，網膜下液あるいは虹彩，角膜擦過物などの眼組織などがある．いずれも少量であるが，PCR法の検体としては十分に行える．また，検体を凍結しておけば，後日利用することもできるので，疑わしい検体は凍結保存しておき再利用できる．

最大の留意点として，この反応の成否は，増幅対象DNAとプライマーの塩基配列，サイクル中の各設定温度・時間などに依存する．それらが不適切な場合，無関係なDNA配列を増幅したり，あるいは増幅が見られないことがある．また，微量検体の場合は従来の定性PCR法では多くの項目の核酸を網羅的には検査できない．さらに，その病巣からPCR法で特定のDNAが検出されたとしても，その陽性という結果はPCR法の感度がよいためにコンタミネーションなどが避けられないことから，偽陽性が含まれる場合があるので注意が必要である．

RT-PCR法

逆転写酵素-ポリメラーゼ連鎖反応法の略．RT-PCR法はcDNAクローニングや遺伝子発現解析に有用な手法である．細胞や組織からRNAを抽出した後，プライマーDNA，逆転写酵素によりcDNAを合成する．このcDNAをテンプレートにして上記のような一般的なPCR法を行う．cDNA合成からPCRまでを連続して行う方法（one-step法）と，cDNA合成とPCRのステップを別途行う方法（two-step法）とがある．RT-PCR法を使う利点は，DNAは余分な情報も含まれているが，スプライシングがされた後のRNAは使用する情報しか含まれていないので，特定したい目的の蛋白構造情報が解析できる点である．最大の欠点は，RNAの不安定さにある．手技が安定した熟練の人ではないと結果の信頼性に欠ける．

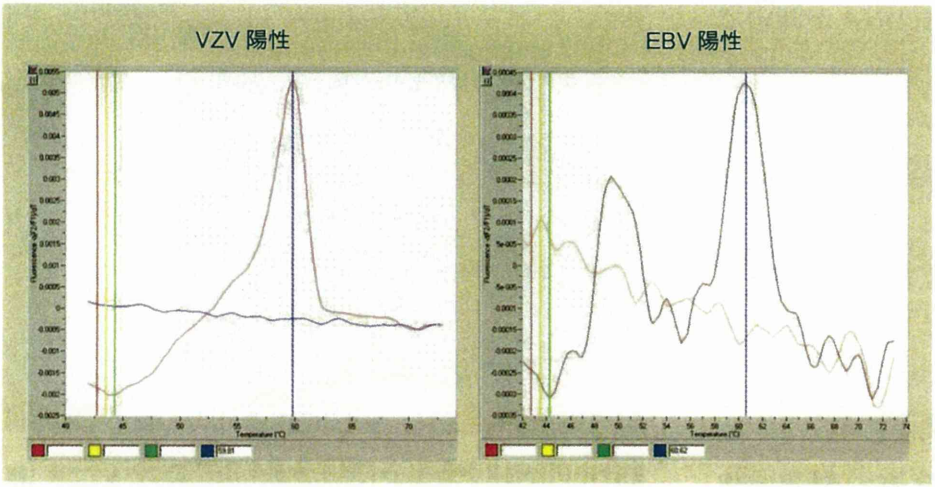


2 multiplex PCR(多項目迅速PCR検査)法

multiplex PCRは，数種類のウイルスなどの外来性抗原を同時に迅速に検出できる新しいPCR検査システム．眼局所検体からDNAを抽出後，Accuprime Taqを用いてそれぞれの特異的プライマーを混合し，ライトサイクラーキャピラリーを用いてPCRを行なう．PCR反応後，ハイブリダイゼーションプローブの混合液とPCR産物を混合し，融解曲線解析を行う．これらはTm値(melting temperature, 融解温度)が重ならないように設定したプローブによってウイルスの種類を判定する．

multiplex PCR法

近年，開発されたのが多項目迅速PCR検査（multiplex PCR法）である（2）[1-3]．multiplex PCR法の最大の特徴は，数種類のウイルスなどの外来性抗原を同時に迅速に検出できることである．以前の一般的に行われていたPCR法のようなゲル内のバンド検出で判定するのではなく，融解曲線で陽性か陰性かの判定を行う（3）．曲線が大きい場合，ウイルス量が多いことがわかり半定量できる利点がある．サンプル調整からPCR法にかかる所要時間はわずか2時間弱と迅速で，場合によっては10項目以上の外来性抗原DNAが陽性か陰性か判定できる．その他の利点は，眼表面炎症性疾患（角膜炎，結膜炎など）の涙液検体は複数の外来性抗原が検出される可能性があり，このmultiplex PCR法は有用と思われる．欠点は，複



3 multiplex PCRを施行した代表症例

融解曲線カーブで陽性か陰性かの判定を行う．この症例は虹彩毛様体炎の炎症が激しく前房蓄膿と前部硝子体混濁まで出現していた．その硝子体液からVZV-DNAが検出され，VZV関連虹彩毛様体炎と診断した．また同検体からEBV-DNAも検出されていた．VZVはTm値が60℃で曲線が検出されるように設定していて，別セットに含まれているEBVは61℃で曲線のピークが検出されるように設定している．同検体の他のヘルペスウイルスDNA（HSV-1，HSV-2，CMV，HHV-6，HHV-7，HHV-8）はすべて陰性であった．

数のPCR反応が同時に一つの反応系で進むようにするため，プライマーの設定，プライマー混合比の複雑な検討が必要であり，技術と時間を要する．また，陽性の外来性抗原種はTm値によって判定されるため，検出するTm値はハイブリプローブのTm値に依存している．このTm値の算出が複雑で正確な設計には多くの作業が必要となる．

real-time PCR法

近年，核酸の量を定量化するPCR検査法，real-time PCR法が出現した．サンプル調整からPCRにかかる所要時間はおおよそ5時間で，DNA量が定量的に判明することが最大の魅力である（⇒**Point!**）．欠点は，PCR機器の手技の難しさと定量PCR法で得られる数字の解釈である．たとえば，特発性ぶどう膜炎患者の前房水からVZV-DNAが1.2×10^3 copies/mL検出された場合，このウイルス量がどこまで眼内炎症の病態にかかわっているのか，などである．定量PCR法で得られる数字はあくまでもウイルスゲノムのコピー数に関する情報であり，ウイルスの感染性とは直接的な関係はない．自分たちで症例を重ねて検出感度を設定する必要がある．

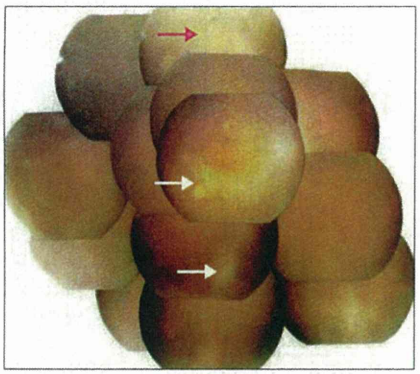
broad-range PCR

PCR法を用いた検査の利点として，感染性ぶどう膜炎や眼内炎を否定する目的で使用できる．最近では，ウイルスだけではなく，broad-range PCR法という細菌や真菌の共通保存領域をPCRで増幅させる方法が報告されている[4]．細菌は16Sリボゾーム RNA領域，真菌は18Sリボゾーム RNA領域を増幅させる．これにより何の菌までかはわからなくても，これらの感染を否定することができ，治療の中心がステロイドのぶどう膜炎分野ではこれらのPCR法が広く使用されるようになってきている．理論的にはすべての外来性抗原をPCR法で検出することが可能で今後は眼科関連性のある外来性微生物をすべて網羅できる検査システムの開発が待たれる．

眼科領域での臨床診断への実際の使用

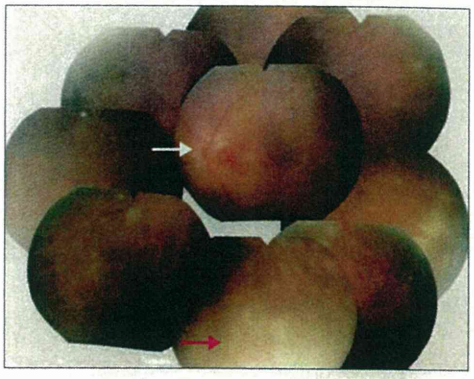
従来の一般的な眼の検体を用いた検査，たとえばウイルスでは抗体測定や蛍光抗体法，細菌や真菌では培養やスメアなどで，これらの検査では確定診断をつけるのが困難な症例がある．

ヒトヘルペスウイルス（human herpesvirus：HHV）は，急性網膜壊死をはじめ多くのぶどう膜炎の原因ウイルスとして知られている．急性網膜壊死患者の前房水や硝子体液からは，高率に単純ヘルペスウイルス〔herpes simplex virus：HSV（HSV-1またはHSV-2），**4**〕，あるいは，水痘・帯状疱疹ウイルス（varicella-zoster virus：VZV）特異的なDNAがPCR法により検出される（**5**）．われわれは急性網膜壊死患者の眼内液から，PCR法により，上記ウイルスに特異的なDNAを検出した[1]．これらの症例では，このPCRの結果よりHSVまたはVZVが眼内炎症の主たる原因と



4 HSV-2が検出された急性網膜壊死の1例

原因不明のぶどう膜炎，網膜血管炎で紹介，受診．網膜血管に沿った滲出斑（⇨）と眼底周辺に壊死病巣（→）が見られている．real-time PCR 法で前房水から HSV-2-DNA が 1.1×10^6copies/mL と高コピー数検出された．multiplex PCR 法によるスクリーニング PCR 法では他のヘルペスウイルスはすべて陰性であった．



5 VZVが検出された急性網膜壊死の一例

初診時，視神経乳頭出血（⇨），硝子体混濁，周辺部に淡い滲出斑（網膜壊死病巣，→）が見られていた．外来時での前房水検査で VZV-DNA が陽性で，そのコピー数が 7.3×10^4 copies/mL であった．その後の手術時の硝子体検体からも VZV-DNA が 5.6×10^6copies/mL と高コピー数検出されていた．

なぜreal-time PCR法が近年盛んに行われるようになったのか

Point!

real-time PCR 法はどの分野でも最も積極的に行われるようになった検査法の一つである．経過中に何度か検体を採取できればこの PCR 法を用いてその経過中の DNA コピー数が把握できたり，また，治療前に局所の抗原 DNA 増幅コピー数を把握できるために，実際の治療薬の量の決定の参考にもなる．われわれは，水痘・帯状疱疹ウイルス（VZV）虹彩毛様体炎の前房水から高コピー数の VZV-DNA が検出されその眼局所のウイルス量と虹彩萎縮や麻痺性散瞳などの組織破壊が相関し，早期診断が重要であることを報告している[3]．

なっているといえる．また，サイトメガロウイルス（CMV）網膜炎患者の眼内液からはCMV-DNAが検出される．CMV眼感染症は後天性免疫不全症候群患者の日和見感染症のなかでは高頻度で出現すること，また臓器移植や，悪性腫瘍治療中に免疫抑制薬を使用する頻度が増えるにつれ，注意を要する．

ヘルペスウイルス属は，原因不明の虹彩毛様体炎を引き起こしていると考えられる．再発性で片眼性の虹彩炎を起こし，中等度の眼圧上昇を伴い，経過中に，虹彩色素脱出，麻痺性散瞳，あるいは虹彩後癒着を伴う虹彩炎では，ヘルペスウイルスの関与が疑われる．多くの場合，ステロイド点眼単独投与では治療に抵抗性する．実際，このような症例の前房水から，PCR法により，HSVやVZV-DNAを検出することができる[2,3]．これらの症例では，アシクロビル局所投与（ゾビラックス®眼軟膏）やバラシクロビル内服の投与を併用することにより，治癒させることが可能である．

その他に，ヘルペスウイルスの関与が疑われている疾患としては，角膜内皮炎，Posner-Schlossman症候群などがあげられる．最近では，Posner-Schlossman症候群類似の高眼圧，軽度の虹彩炎患者のなかにCMV-DNAが検出されたとの報告がある[2]．臨床所見的にはPosner-Schlossman症候群と酷似しており，その鑑別は困難である．また，角膜内皮炎でも前房水からCMV-DNAが検出される報告もある．その他，PCR法での報告がある眼科関連ウイルスはEpstein-Barrウイルス（EBV），HHV-6[5]，レトロウイルスのHTLV-1などがあげられる．

■引用文献

1. 杉田　直，ほか：急性網膜壊死患者眼内液の多項目迅速ウイルスPCRおよびリアルタイムPCR法によるヘルペスウイルス遺伝子同定．日眼会誌 2008; 112: 30-38.
2. Sugita S, et al: Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. Br J Ophthalmol 2008; 92: 928-932.
3. Kido S, et al: Association of varicella-zoster virus (VZV) load in the aqueous humor with clinical manifestations of anterior uveitis in herpes zoster ophthalmicus and zoster sine herpete. Br J Ophthalmol 2008; 92: 505-508.
4. Chiquet C, et al: Eubacterial PCR for bacterial detection and identification in 100 acute postcataract surgery endophthalmitis. Invest Ophthalmol Vis Sci 2008; 49: 1971-1978.
5. Sugita S, et al: Identification of human herpesvirus 6 in a patient with severe unilateral panuveitis. Arch Ophthalmol 2007; 125: 1426-1427.

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Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR

Sunao Sugita,[1] Norio Shimizu,[2] Ken Watanabe,[2] Miki Katayama,[2] Shintaro Horie,[1] Manabu Ogawa,[1] Hiroshi Takase,[1] Yoshiharu Sugamoto,[1] Manabu Mochizuki[1]

[1]Department of Ophthalmology & Visual Science, Medical Research Institute, Tokyo Medical and Dental University Graduate School of Medicine and Dental Sciences, Tokyo, Japan
[2]Department of Virology, Medical Research Institute, Tokyo Medical and Dental University Graduate School of Medicine and Dental Sciences, Tokyo, Japan

Correspondence to
Dr Manabu Mochizuki, Department of Ophthalmology & Visual Science, Tokyo Medical and Dental University Graduate School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan; m.manabu.oph@tmd.ac.jp

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ABSTRACT

Aim To measure the bacterial genome in ocular fluids and to analyse the clinical relevance of infectious endophthalmitis.

Methods Nineteen ocular fluid samples (eight aqueous humour and 11 vitreous fluid samples) were collected from 19 patients with suspected bacterial endophthalmitis. Fifty ocular samples from uveitis patients were also collected along with 40 samples from patients without ocular inflammation and used as controls. Bacterial ribosomal DNA (16S rDNA) was measured by a quantitative PCR assay.

Results Bacterial 16S rDNA was detected in patients with clinically suspected bacterial endophthalmitis (18/19, 95%). With the exception of one case, high copy numbers of bacterial DNA were detected (1.7×10^3—1.7×10^9 copies/ml) in these patients. There were 10 samples (53%) with positive bacterial cultures while there were nine samples (47%) with positive Gram-staining. Real-time PCR detected bacterial 16S rDNA in three (6%) of the 50 samples from the control uveitis patients. In addition, none of the samples from the control patients without intraocular inflammation were positive.

Conclusions Quantitative broad-range PCR of bacterial 16S rDNA is a useful tool for diagnosing bacterial endophthalmitis.

INTRODUCTION

Bacterial infectious endophthalmitis occurs due to exogenous infections, such as those arising from trauma and intraocular surgery, or from endogenous infections, such as systemic infectious disorders. Previous studies have used PCR to demonstrate the presence of bacterial DNA in the ocular fluids in patients with infectious endophthalmitis.[1–10] PCR has often been used to provide evidence of bacterial involvement in the eyes with suspected intraocular infections.[8] These suspected infections include idiopathic endophthalmitis and uveitis. Recent advances in molecular biology along with the use of real-time PCR have made it possible to determine quantitative measurements of the viral load associated with viral diseases in the eye.[11–13] Several studies have recently reported finding the bacterial ribosomal RNA gene (16S rDNA) in the ocular fluids of patients with infectious endophthalmitis.[4 8 10] With primers of the bacterial 16S rRNA gene, broad-range PCR can be used to detect the presence of bacteria within the samples. In endophthalmitis patients with previous intravitreal administration of antibiotics, PCR methodology has been shown to be more effective than bacterial cultures in detecting bacterial DNA in the ocular fluids.[10] However, even broad-range PCR has not been able to determine quantitative information for the bacterial genome in the ocular sample.

In the present study, after collecting ocular samples from patients with suspected intraocular infections, which included bacterial infectious endophthalmitis, we attempted to detect and then measure the bacterial genome using real-time quantitative PCR with primers for 16S rDNA amplifications.

MATERIAL AND METHODS

Subjects

Based upon medical history and clinical observations, 69 patients with endophthalmitis and uveitis were consecutively enrolled in a prospective study that was conducted from 2008 to 2009 at the Tokyo Medical and Dental University Hospital. Samples of aqueous humour and vitreous fluids were collected from all patients. Nineteen patients (19 eyes: eight aqueous humour and 11 vitreous fluids) had bacterial infectious endophthalmitis. Of these 19 patients, six had acute postoperative endophthalmitis, four had late postoperative endophthalmitis, one had post-traumatic endophthalmitis, five had endogenous endophthalmitis, two had keratitis-associated endophthalmitis, and one had endophthalmitis after intravitreal injections of bevacizumab.

The second patient group was also a prospective study, and 50 ocular samples were collected from various patients with uveitis. The underlying pathology included idiopathic uveitis (n=21), herpetic keratouveitis (n=3), herpetic anterior iridocyclitis (n=3), acute retinal necrosis (n=5), cytomegalovirus retinitis (n=2), toxoplasmosis (n=3), toxocariasis (n=2), sarcoidosis (n=2), HTLV-1-associated uveitis (n=1), toxic lens syndrome (n=1), *Candida* endophthalmitis (n=2) and intraocular lymphoma (n=5). In this study, fungal endophthalmitis cases such as *Candida* endophthalmitis were classified as being part of this patient group. All the patients displayed active intraocular inflammation at the time of sampling.

In addition to the patient groups, we also analysed samples from a control group. These patients were enrolled in this prospective study in 2009. Forty samples (20 aqueous humour and 20 vitreous fluids) were collected from patients who did not have any type of ocular inflammation (age-related cataract, macular oedema secondary to branch retinal vein occlusion, retinal detachment, idiopathic macular hole or idiopathic epiretinal membrane).

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For the ocular sampling (asepsis), the following procedures were performed in all subjects. In all of the eyes that were sampled, the ocular surfaces, including the conjunctival sacs, were rinsed once with an aqueous povidone iodine solution. Subsequently, all of these eyes were then rinsed once with a balanced-salt solution. A 0.1 ml aliquot of aqueous humour was collected aseptically in a syringe with a 30 G needle. Half of the sample was then transferred into a pre-sterilised microfuge tube and used for PCR.

In patients with endophthalmitis/uveitis who were undergoing vitreous surgery, uncontaminated non-diluted vitreous fluid samples (0.5–1.0 ml) were collected during diagnostic pars plana vitrectomy (PPV). Immediately after collection, 100 µl of the sample was transferred into a pre-sterilised microfuge tube and used for PCR. None of the asepsis samples used for analysis came from patients being given systemic antibiotics or from patients who were receiving intraocular antibiotic injections.

Conventional microbiological investigations

The Bacteria Work Station of the Tokyo Medical and Dental University Hospital processed all specimens (aqueous humour and vitreous fluids) within 1 h after the sample collection, with standard methods followed for the isolation and identification of the aerobic and anaerobic bacterial cultures. The culture methods followed conventional techniques that have been previously published.[14 15] Cultures were incubated for up to 7 days, with those lacking growth designated as culture-negative. Cytospin smears of the specimens were stained using Gram's method for detection of bacteria.

Quantitative PCR

DNA was extracted from samples using a DNA minikit (Qiagen, Valencia, California, USA) installed on a Robotic workstation for automated purification of nucleic acids (BioRobot E21, Qiagen). The real-time PCR was performed using Amplitaq Gold and the Real-Time PCR 7300 system (Applied Biosystems, Foster City, California, USA). Primers and probes of bacterial 16S rDNA and the PCR conditions are described elsewhere.[16] The sense primer (Bac349F) was 5'-AGGCAGCAGTDRGGAAT-3' and the antisense primer (Bac806R) was 5'-GGACTACYVGGGTATCT-AAT-3'. The TaqMan probe (Bac516F) was 5'-FAM-TGCCAGC-AGCCGCGGTAATACRDAG-TAMRA-3'. Products were subjected to 50 cycles of PCR amplification, with cycling conditions set at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Amplification of the human β-globulin gene served as an internal positive extraction and amplification control. Bacterial copy number values of more than 100 copies/ml in the sample were considered to be significant.

Sensitivity of TaqMan real-time PCR

To confirm the real-time PCR assay sensitivity, the 458 bp fragments were amplified from the DNA of *Staphylococcus aureus* (NBRC 12732) with Bac349F and Bac806R. The PCR fragments were inserted into the pGEM cloning plasmid with the pGEM T-Easy Vector Cloning System I kit (Promega, Tokyo, Japan). The plasmid was digested with restriction enzyme ScaI. Linearised plasmid was controlled by gel electrophoresis and quantified by using the Smart Ladder DNA size and mass marker (Wako, Tokyo, Japan) and the OD260 measurement. Standard curves were constructed from serial 10-fold dilutions of linearised plasmid DNA with 10 ng/µl MS2 RNA (Basel, Roche, Switzerland). The detection limit and standard range of the TaqMan real-time PCR were determined by using serial 10-fold dilutions of linearised plasmid. The standard range of DNA was linearly quantified from one to nine log DNA copies, with a detection limit of 10 copies. The negative control (nuclease-free water) was not detected.

PCR FOR 16S rRNA GENE AND SEQUENCE ANALYSIS

PCR mix (50 µl volumes) was prepared from Low-DNA AmpliTaq Gold DNA polymerase LD (Applied Biosystems). The mix comprised dATP, dGTP, dCTP, dTTP, 2 mM $MgCl_2$ and 1×Gold buffer, along with each of the primers (500 nM) (forward primer fD1-AGAGTTTGATCCTGGCTCAG; reverse primer rp2-ACGGCTACCTTGTTACGACTT).[17]

Template DNA, 1.25U of AmpliTaq Gold DNA polymerase LD (Applied Biosystems), and nuclease-free water were added to the sample. The PCR assay was performed using the Takara Thermal Cycler TP-400 (Takara Bio Inc., Shiga, Japan). The cycling conditions used were: 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, 42°C for 30 s, and 72°C for 4 min. Gel electrophoresis was performed using a 0.8% agarose gel (Takara Bio Inc.) in 40 mmol/l Tris, 1 mmol/l EDTA for 30 min at 100 V, followed by ethidium bromide staining. Before cycle sequencing, amplicons were purified using the Qiaquick PCR purification kit (Qiagen) according to the manufacturer's protocol. Cycle sequencing was performed by forward and reverse priming using the Big Dye v3.1 Terminator Reaction kit (Applied Biosystems). The PCR assay was performed using a Perkin Elmer 9700 with cycling conditions set at: 95°C for 30 s, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Electrophoresis was conducted in a 3130xl genetic analyser (Applied Biosystems).

We used the DNA sequence analysis to examine patients suspected of having bacterial endophthalmitis (patient samples that only had high amounts of total DNA and detected high copy numbers of bacterial 16S rDNA). Basic local alignment search tool (BLAST) analysis was used to examine the DNA sequences. The 16S rDNA sequences obtained were compared with those available in the GenBank BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Using a previously published method,[18] positive identification of the species level was defined as identification of a 16S rDNA sequence that had 99% similarity or greater with that of the GenBank BLAST strain sequence.

Prevention of bacterial contamination

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. The preparation was performed on a laminar flow workbench and employed single-use aliquots of reagent and dedicated pipettes. Microfuge tubes and mineral oil aliquots were carefully sterilised prior to use.

RESULTS

Our initial PCR results indicated that bacterial 16S rDNA was positive in 18 ocular fluids of the clinically suspected bacterial endophthalmitis patients (18/19, 95%, table 1). These positive patients had high copy numbers of 16S rDNA ranging from 1.7×10^5 to 1.7×10^9 copies/ml, which indicated the presence of bacterial infection. In the one PCR-negative case (case 16 in table 1), PCR did not detect any bacterial genome in the vitreous fluid (<100 copies), although *Klebsiella pneumoniae* was detected in the biopsy sample of the liver abscess.

In the conventional bacterial cultures, 10 (53%) out of the 19 samples were positive (table 1). In addition, positive Gram staining was found in nine (47%) out of these samples. There were only two patients (cases 2 and 4 in table 1) that received

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Table 1 Detection of bacterial 16S rDNA in suspected bacterial endophthalmitis and uveitis

Case	Diagnosis	Sample	Bacterial 16S rDNA	Culture	Smear	BLAST analysis	Treatment
1	Postoperative (acute)	AH	2.8×10^8 copies/ml	*Staphylococcus* spp.	Negative	nt	PPV, IAI, SA
2	Postoperative (acute)	VF	1.5×10^8 copies/ml	Negative	Negative	nt	PPV, IAI, SA
3	Postoperative (acute)	AH	1.5×10^6 copies/ml	*Staphylococcus epidermidis*	G (+)	*Staphylococcus epidermidis*	PPV, IAI, SA
4	Postoperative (acute)	VF	7.5×10^6 copies/ml	Negative	Negative	nt	PPV, IAI, SA
5	Postoperative (acute)	VF	9.0×10^7 copies/ml	Negative	G (+)	nt	PPV, IAI, SA
6	Postoperative (acute)	VF	1.9×10^7 copies/ml	*Streptococcus sanguinis*	G (+)	*Streptococcus sanguinis*	PPV, IAI, SA
7	Postoperative (late)	VF	8.1×10^7 copies/ml	Negative	Negative	*Bradyrhizobium elkanii*	PPV, IAI, SA
8	Postoperative (late)	AH	1.7×10^3 copies/ml	Negative	Negative	nt	SA
9	Postoperative (late)	AH	3.9×10^4 copies/ml	Negative	Negative	nt	SA
10	Postoperative (late)	AH	8.6×10^4 copies/ml	*Pseudomonas aeruginosa*	G (−)	nt	PPV, IAI, SA
11	Post-traumatic	VF	1.4×10^6 copies/ml	*Enterococcus faecalis*	G (+)	*Enterococcus faecalis*	PPV, SA
12	Endogenous	VF	1.3×10^7 copies/ml	*Pseudomonas* sp.	G (−)	*Pseudomonas* sp. PR	PPV, IAI, SA
13	Endogenous	VF	1.7×10^9 copies/ml	*α-Streptococcus*	G (+)	*Streptococcus mitis*	PPV, IAI, SA
14	Endogenous	AH	1.1×10^4 copies/ml	Negative	Negative	nt	IAI, SA
15	Endogenous	VF	5.5×10^6 copies/ml	*Staphylococcus aureus*	Negative	*Staphylococcus aureus*	PPV, IAI, SA
16	Endogenous	AH	<100 copies/ml	Negative	Negative	nt	PPV, IAI, SA
17	Keratitis	AH	3.1×10^6 copies/ml	*Streptococcus pneumoniae*	G (+)	*Streptococcus pneumoniae*	IAI, SA
18	Keratitis	VF	6.8×10^4 copies/ml	Negative	Negative	nt	IAI, SA
19	Intravitreal injection*	VF	1.8×10^6 copies/ml	*Streptococcus oralis*	G (+)	*Streptococcus* sp.	PPV, IAI, SA
20	Idiopathic uveitis	AH	1.4×10^3 copies/ml	Negative	nt	nt	IAI
21	Idiopathic uveitis	VF	6.1×10^4 copies/ml	Negative	Negative	nt	SA
22	CMV retinitis	AH	4.2×10^3 copies/ml	Negative	nt	nt	IAI, SA

AH, aqueous humour; BLAST, basic local alignment search tool; CMV, cytomegalovirus; IAI, intravitreal antibiotic injection; nt, not tested; PPV, pars plana vitrectomy; SA, systemic antibiotics; VF, vitreous fluids.
Using broad-range quantitative PCR, bacterial 16S rDNA could be detected in the ocular samples of the suspected bacterial endophthalmitis cases (18/19, 95%). Broad-range quantitative PCR was also used to measure the bacterial genome in the ocular samples collected from the uveitis patients (n=50) and from the three patients (6%) that were positive.
*Bacterial endophthalmitis after intravitreal injections of bevacizumab.

intravitreal injections of antibiotics prior to the PCR analysis. As shown in table 1, after examinations that included PCR, all patients received antibiotics (systemic and/or local medications).

With the exception of three out of the 50 uveitis patients, real-time PCR indicated the patients were negative for the bacterial 16S rDNA. Details for the three exceptions are shown in table 1.

The 16S rDNA was detected in two patients with idiopathic uveitis and one with cytomegalovirus (CMV) retinitis. Clinically, all of these patients were diagnosed with unilateral uveitis. Bacterial cultures were negative in all of the tested samples. In addition, bacterial 16S rDNA was not detected in any of the 40 control samples collected from the patients without ocular inflammation.

To identify the specific bacterial species, we used BLAST analysis to examine some of the bacterial infectious endophthalmitis patients. Analysis was only possible when the patient's samples had high amounts of total DNA and there was a detected high copy number of the bacterial 16S rDNA. As summarised in table 1, BLAST analysis identified *Staphylococcus epidermidis* (case 3), *Streptococcus sanguinis* (case 6), *Bradyrhizobium elkanii* (case 7), *Enterococcus faecalis* (case 11), *Pseudomonas* sp. *PR* (case 12), *Streptococcus mitis* (case 13), *Staphylococcus aureus* (case 15), *Streptococcus pneumoniae* (case 17) and *Staphylococcus* sp. (case 19). The results of the BLAST analysis were identical to the results of the bacterial culture with the exception of case 7, who was found to have a negative culture. However, even though the bacterial examinations such as bacterial cultures and smears were negative in this patient with late postoperative endophthalmitis, broad-range real-time PCR analysis of the vitreous sample yielded positive results (8.1×10^7 copies/ml). In the present study, once we were able to determine the bacterial species via the BLAST analysis and conclusively diagnose bacterial endophthalmitis, we were then able to begin treatment with antibiotics.

Case report

As seen in table 1, case 7 was a 75-year-old man who was referred to the uveitis clinic at our hospital during July 2007 due to keratic precipitates, cells and fibrin in the anterior chamber along with hypopyon and anterior vitreous opacity in his right eye (figure 1). The patient had undergone cataract surgery in his right eye 1 year prior to being seen in our clinic. Although visual acuity of his right eye at the time of his initial presentation to our clinic was 0.8, 2 months later, his visual acuity was less than 0.1. A vitreous sample was collected during the pars plana

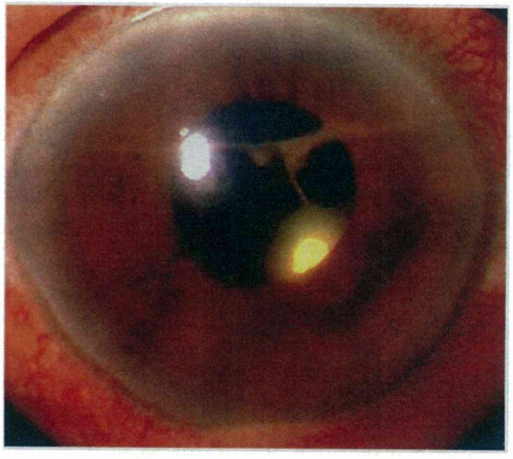

Figure 1 Case 7 (late postoperative endophthalmitis). Slit-lamp photograph in suspected bacterial endophthalmitis. In the right eye, cyclitic membrane, height of the hypopion, and severity of vitritis were seen. In this patient, broad-range quantitative PCR revealed a high copy number of the bacterial genome (8.1×10^7 copies/ml). Basic local alignment search tool (BLAST) analysis detected *Bradyrhizobium elkanii*.

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vitrectomy. While bacterial culture and the Gram-staining of the vitreous sample were negative, broad-range and real-time PCR detected 8.1×10^7 copies/ml of bacterial 16S rDNA (table 1). In addition, the BLAST analysis detected *Bradyrhizobium elkanii*. After the patient was given an intravitreal antibiotic injection (vancomycin and ceftazidime) and systemic antibiotics (levofloxacin), inflammation in his right eye completely disappeared. After receiving treatment, visual acuity in his right eye recovered to 0.9 and there was no severe intraocular tissue damage noted.

DISCUSSION

In the present study, with the exception of one patient, we detected bacterial 16S rDNA in all of the cases that were clinically suspected to have bacterial endophthalmitis. In these patients, high copy numbers of the bacterial DNA were detected, which indicated the presence of a bacterial infection. In the single patient who was suspected of having infectious endophthalmitis but had no bacteria in the ocular sample, *K. pneumoniae* was detected by biopsy culture for liver infection. Thus, we were ultimately able to diagnose the patient as having endogenous endophthalmitis.

On the other hand, conventional microbiological investigations of the ocular fluid samples, such as bacterial cultures and smears, were negative in about one-half of these patients. Only three of the 50 samples collected from the patients with other clinical entities of uveitis were positive for the broad-range real-time PCR analyses of the bacterial 16S rDNA. In addition, no bacterial 16S rDNA was detected in any of the samples from the control patients without ocular inflammation.

The potential advantage of using PCR is that minute numbers of bacteria can be detected from the very small specimens that are required for the analysis. Chen *et al*[19] developed this PCR detection method for the eubacterial genome based on the conserved regions of the 16S rRNA sequence (16S rDNA) of *Escherichia coli*. As the universal primers chosen from 16S rDNA have a large amount of sequence information and highly conserved regions of the gene, primers can be synthesised for a wide variety of bacteria. In addition, the eubacterial primers used had both a high specificity and sensitivity, which was comparable to previous studies.[1 3] Hykin *et al*[1] examined 29 control vitreous samples and found four that were positive for the eubacterial genome using PCR. In a further study by Therese *et al*,[3] only a single control sample (5%) was found using the eubacterial-based PCR. In the present study, we did not detect any bacterial 16S rDNA (<100 copies/ml) in any of the samples from the control non-infectious patients when using our broad-range real-time PCR. Thus, another potential advantage of our PCR system is that it provides quantitative information for the bacterial infection. In the present study, we found false positive results (1–100 copies/ml) in only two control samples that we tested, a result that could be due to contamination caused by the conjunctival ocular flora present during the collection of the samples. Other possible causes of the contamination might be related to technical errors that occurred during the PCR preparation or perhaps due to bacterial exposure when collecting the ocular sample.

In cases of bacterial infectious endophthalmitis, it is often difficult to differentiate between inflammation caused by non-infectious and infectious agents. For example, to determine the cause of postoperative inflammation in the eye, we must consider many different possibilities, such as surgical manipulation, toxic lens syndrome, recurrent uveitis (especially if the patient has a previous history) or bacterial endophthalmitis. In the past, microbiological investigations of the ocular fluids have often failed to detect the infectious agent in bacterial endophthalmitis, resulting in a clinical dilemma regarding therapy. Deciding to use antibiotics and steroids necessitates determining whether an inflammation is infectious or sterile. Therefore, an aetiological diagnosis is essential in such cases. The use of PCR with universal eubacterial primers, which possesses broad specificities for all Gram-positive and -negative bacteria, has been recently found to be much more useful for detecting the eubacterial genome in ocular samples of postoperative endophthalmitis cases compared to the routine microbiological investigations.[2 5 5 6 8–10] In the present study, our broad-range real-time PCR for the eubacterial genome showed high correlation with the bacteriologically positive samples. This suggests that bacteriologically negative samples may include the bacterial genome. In a recent report by the French Institutional Endophthalmitis Study Groups, eubacterial PCR was found to be much more effective than bacterial cultures in detecting bacteria in vitreous samples from patients with previous intravitreal administration of antibiotics.[10] Although the previous administration of antibiotics in the PPV vitreous fluids may inhibit bacterial growth, it is assumed that PCR may still be able to detect bacterial DNA of either living or killed bacteria.

As revealed in this study, real-time PCR found only three (6%) of the 50 ocular samples from patients with unilateral uveitis to be positive. However, high copy numbers of bacterial DNA were detected in these uveitis patients, which included idiopathic uveitis (n=2) and cytomegalovirus retinitis (n=1). Endophthalmitis and uveitis positive cases with low quantification of DNA (eg, 1×10^5–1×10^4 copies/ml) cannot be differentiated according to the number of copies. Although topical or systemic steroids were administered for long periods in the idiopathic uveitis patients, the inflammation remained uncontrolled. It has also been reported that viral PCR has found cytomegalovirus DNA in the eyes of cytomegalovirus retinitis cases.[15] When these patients were given intravitreal administration of an antiviral injection (Ganciclovir), an anterior vitreous opacity was subsequently observed. There were three cases that received antibiotics (intravitreal injection and/or systemic) in our study and the intraocular inflammation, such as vitreous opacity, was well controlled by this antibiotic therapy. Although bacterial DNA amplification in such cases usually suggests contamination, antibiotic administration proved to be effective in our study. Thus, the bacterial PCR-based evidence suggests bacterial involvement in eyes that have a suspected intraocular infection. While PCR for eubacterial detection is necessary for rapid and accurate diagnosis in patients suffering from an unknown intraocular inflammatory disorder, it can also be used to accurately determine samples that are not infected. In our study we found 47 samples (94%) that had negative PCR results. Overall, our results suggest that a sensitive and rapid diagnostic test not only allows for confident verification of the diagnosis (non-infectious inflammation vs infection), but also allows for early commencement of specific and appropriate treatment. In addition, PCR analysis is able to exclude bacterial infections as the potential cause of an ocular disorder.

In conclusion, this new PCR system is an excellent diagnostic system for intraocular specimens and can be used as an alternative to further examine specimens determined to be bacteriologically negative by conventional methods. Our study also clearly demonstrated that a new diagnostic PCR system using eubacterial detection with broad-range PCR along with quantitative evaluation with real-time PCR could be extremely useful for detecting bacterial DNA within ocular samples. Recently, Goldschmidt *et al* reported that a new diagnostic test for

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Propionibacteriaceae was designed using TaqMan real-time PCR.[20] Therefore, the ability to be able to collect quantitative information on bacterial infections in the eye should be useful in helping to determine clinical diagnoses and therapeutic follow-ups. Moreover, using a combination of the quantitative PCR method and the BLAST analysis to detect bacterial species is a very valuable tool for diagnosing suspected bacterial endophthalmitis. However, the DNA in 10 of 19 samples could not be sequenced using this technique and thus could not identified, which could potentially limit the clinical usefulness of this technique at the present time. In order for clinicians to be able to obtain bacterial identifications, we may need to consider additional options for the sequence analysis. In addition, in the future we will need to further verify whether this broad-range PCR can detect candidate bacterial DNA including *K. pneumoniae* in bacterial endophthalmitis.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Institutional Ethics Committee of Tokyo Medical and Dental University. The research followed the tenets of the Declaration of Helsinki.

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REFERENCES

1. **Hykin PG,** Tobal K, McIntyre G, *et al*. The diagnosis of delayed post-operative endophthalmitis by polymerase chain reaction of bacterial DNA in vitreous samples. *J Med Microbiol* 1994;**40**:408–15.
2. **Lohmann CP,** Heeb M, Linde HJ, *et al*. Diagnosis of infectious endophthalmitis after cataract surgery by polymerase chain reaction. *J Cataract Refract Surg* 1998;**24**:821–6.
3. **Therese KL,** Anand AR, Madhavan HN. Polymerase chain reaction in the diagnosis of bacterial endophthalmitis. *Br J Ophthalmol* 1998;**82**:1078–82.
4. **Knox CM,** Cevallos V, Margolis TP, *et al*. Identification of bacterial pathogens in patients with endophthalmitis by 16S ribosomal DNA typing. *Am J Ophthalmol* 1999;**128**:511–12.
5. **Lohmann CP,** Linde HJ, Reischl U. Improved detection of microorganisms by polymerase chain reaction in delayed endophthalmitis after cataract surgery. *Ophthalmology* 2000;**107**:1047–51.
6. **Anand AR,** Madhavan HN, Therese KL. Use of polymerase chain reaction (PCR) and DNA probe hybridization to determine the Gram reaction of the infecting bacterium in the intraocular fluids of patients with endophthalmitis. *J Infect* 2000;**41**:221–6.
7. **Okhravi N,** Adamson P, Lightman S. Use of PCR in endophthalmitis. *Ocul Immunol Inflamm* 2000;**8**:189–200.
8. **Okhravi N,** Adamson P, Carroll N, *et al*. PCR-based evidence of bacterial involvement in eyes with suspected intraocular infection. *Invest Ophthalmol Vis Sci* 2000;**41**:3474–9.
9. **Chiquet C,** Lina G, Benito Y, *et al*. Polymerase chain reaction identification in aqueous humour of patients with postoperative endophthalmitis. *J Cataract Refract Surg* 2007;**33**:635–41.
10. **Chiquet C,** Cornut PL, Benito Y, *et al*. Eubacterial PCR for bacterial detection and identification in 100 acute postcataract surgery endophthalmitis. *Invest Ophthalmol Vis Sci* 2008;**49**:1971–8.
11. **Sugita S,** Shimizu N, Kawaguchi T, *et al*. Identification of human herpes virus 6 in a patient with severe unilateral panuveitis. *Arch Ophthalmol* 2007;**125**:1426–71.
12. **Kido S,** Sugita S, Horie S, *et al*. Association of varicella zoster virus load in the aqueous humor with clinical manifestations of anterior uveitis in herpes zoster ophthalmicus and zoster sine herpete. *Br J Ophthalmol* 2008;**92**:505–8.
13. **Sugita S,** Shimizu N, Watanabe K, *et al*. Use of multiplex PCR and real-time PCR to detect human herpes virus genome in ocular fluids of patients with uveitis. *Br J Ophthalmol* 2008;**92**:928–32.
14. **Allen SD.** Anaerobic bacteria. In: Lennete Edwin H, ed. *Manual of clinical microbiology*. 4th edn. Washington DC: American Society for Microbiology, 1985:413–72.
15. **Baron EJ,** Peterson LR, Finegold SM. *Bailey and Scott's diagnostic microbiology*. 9th edn. St Louis: Mosby, 1994:79–136.
16. **Takai K,** Horikoshi K. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Appl Environ Microbiol* 2000;**66**:5066–72.
17. **Weisburg WG,** Barns SM, Pelletier DA, *et al*. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991;**173**:697–703.
18. **Goldenberger D,** Kunzle A. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. *J Clin Microbiol* 1997;**35**:2733–9.
19. **Chen K,** Neimark H, Rumore P, *et al*. Broad range DNA probes for detecting and amplifying eubacterial nucleic acids. *FEMS Microbiol Lett* 1989;**48**:19–24.
20. **Goldschmidt P,** Ferreira CC, Degorge S, *et al*. Rapid detection and quantification of Propionibacteriaceae. *Br J Ophthalmol* 2009;**93**:258–62.

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CLINICAL INVESTIGATION

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

Sunao Sugita · Manabu Ogawa · Shizu Inoue · Norio Shimizu · Manabu Mochizuki

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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.

S. Sugita (✉) · M. Ogawa · S. Inoue · M. Mochizuki
Department of Ophthalmology and Visual Science, Tokyo Medical and Dental University Graduate School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
e-mail: sunaoph@tmd.ac.jp

N. Shimizu
Department of Virology, Medical Research Institute, Tokyo Medical and Dental University Graduate School of Medicine and Dental Sciences, Tokyo, Japan

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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

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

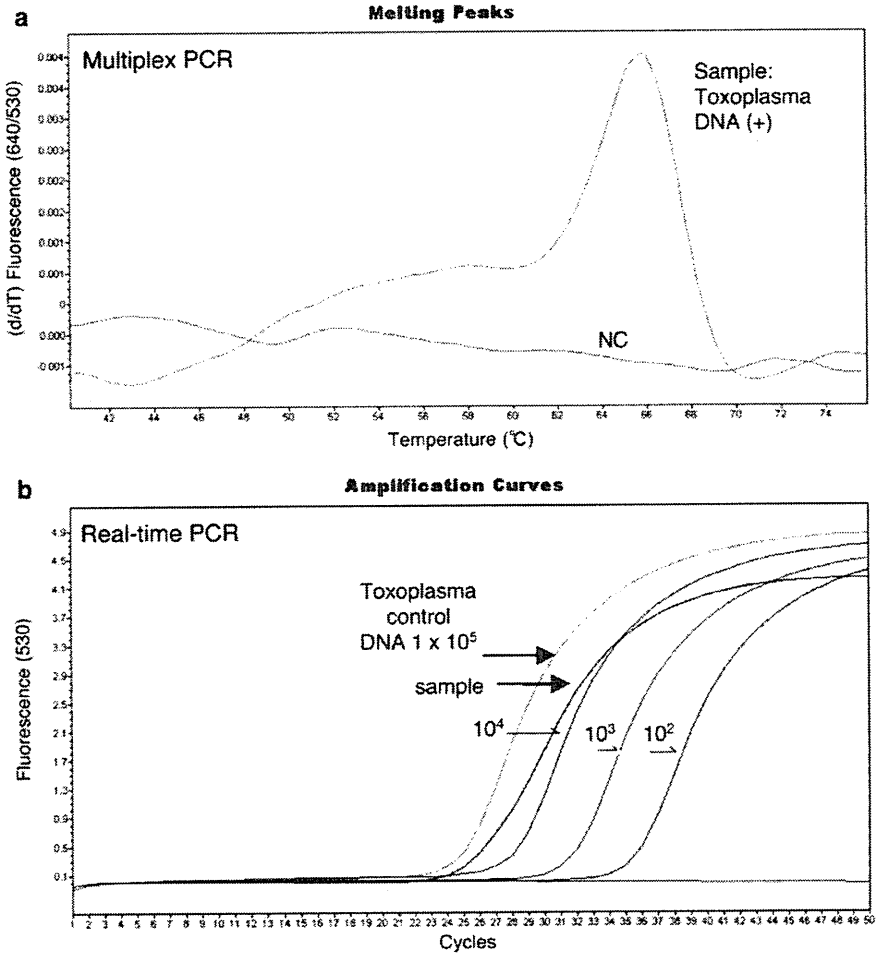

Fig. 1 PCR results for a patient with ocular toxoplasmosis (Case 1 in Table 3). **a** After DNA extraction from the sample, multiplex PCR was performed to screen for *T. gondii*, and for HHV1 to HHV8 using LightCycler capillaries. At 66°C, a significant positive curve was detected, indicating the detection 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,