表1　網羅的迅速微生物検査により得られた成果

1. 急性網膜壊死（桐沢型ぶどう膜炎）検体よりHSV-1，HSV-2，VZV，EBVを検出し，前房水中のウイルス量が薬物療法のみで経過観察するか硝子体手術が必要かの目安になることが示唆された．
2. 難治性ぶどう膜炎の眼内液からHHV6-Aを検出し，本ウイルスがぶどう膜炎発症に関与している可能性を示した．
3. 造血幹細胞移植後の発熱イベントのうち43％にウイルスが関与しており，そのうちの30％が複数ウイルスの同時感染であった．
4. 造血幹細胞移植後のウイルス血症例ではCMV，HHV-6，EBV，BKV，JCVの頻度が高く，CMV，BKVはGVHDの重要度と相関した．
5. 移植後尿路感染症の70％からウイルスが検出され，BKV，JCV，ADV，CMVの順に頻度が高かった．少量のADV陽性でも血尿を生じるが，BKVは10^6 copies/mL以下では無症状の場合が多かった．
6. 造血幹細胞の意識障害患者の髄液検査では，半数からウイルスが検出され，HHV-6脳炎の頻度が最も高かった．

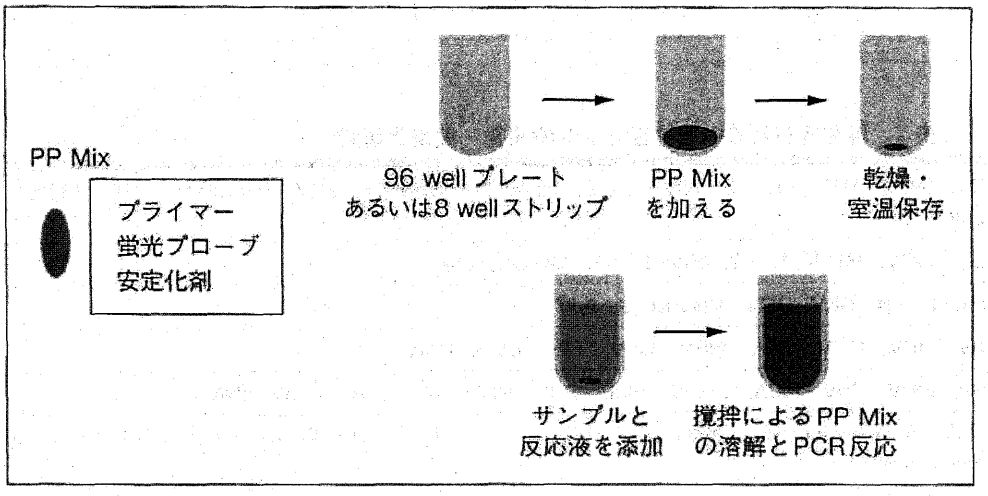

図6a　Ready-to-Use検査プレートの作製と検査実施の概要

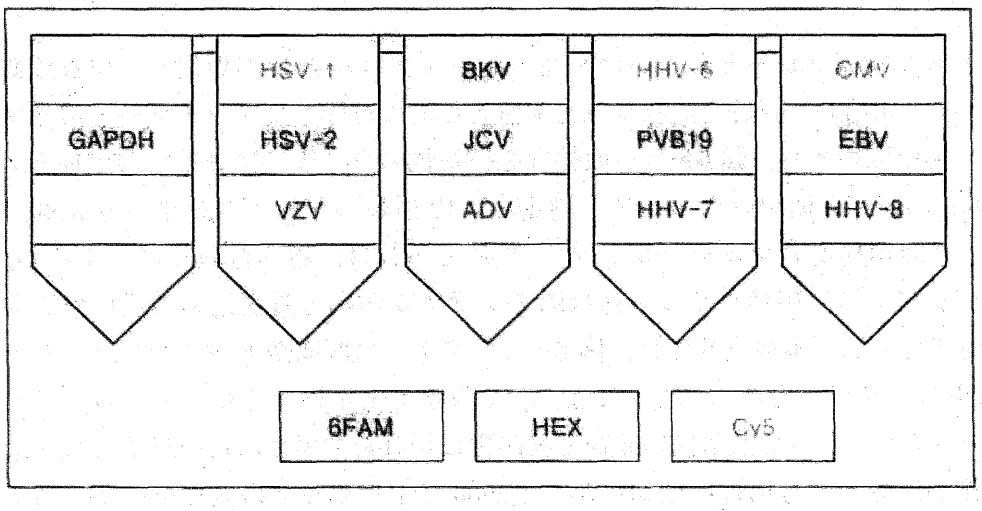

図6b　PP Mixと固相化したReady-to-Use検査ストリップの1例
1 wellで3種類，合計12種類のウイルスの測定ができる（GAPDHはポジティブコントロール，6FAM，HEX，Cy5は蛍光色素）本セットは造血幹細胞移植後の日和見ウイルス感染症の検査用に使用されている．

多い検査項目があるとdNTP等を消費してコピー数が少ない検査項目が陰性化する懸念がある，検査は定性的で定量検査を別途行う必要がある，検査のセットアップが煩雑，キャピラリーの取扱いに習熟していないと破損してコンタミネーションを引き起こすおそれがある，などの欠点がある．筆者の研究室では，これらの欠点を克服したReady-to-Useの検査セットの作製を目指し，図6aに示すような新しい検査系を作製した．

この検査系は96 well型のリアルタイムPCR装置を使用する．各検査項目のプライマーと蛍光

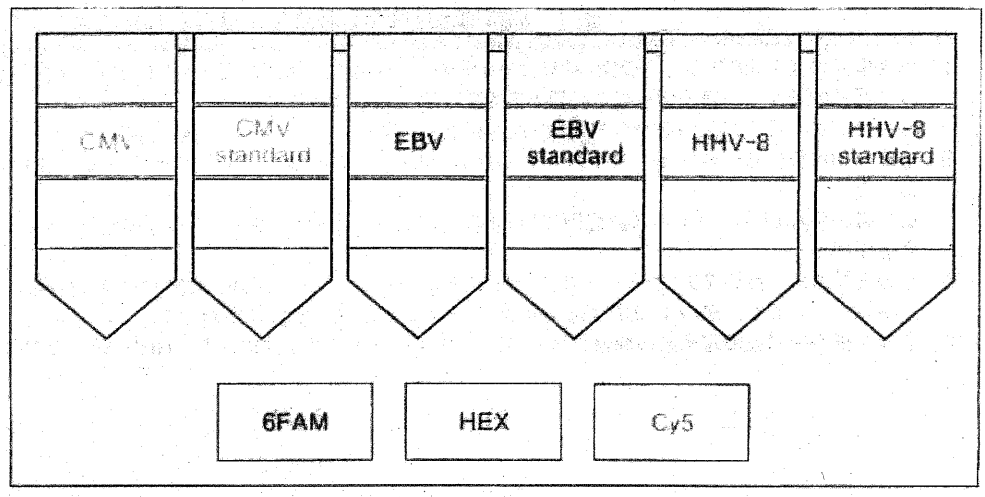

図6c　図6bで陽性が出た場合に使用する定量用ストリップ
ここでは，4番目のwellから陽性シグナルが出た場合に使用する定量用ストリップを示す．スタンダードの値からCt法で陽性項目の定量結果を得ることができる．1 wellで1ウイルスの測定を行うため，複数のウイルスが陽性な場合でも正確に定量ができる．

表2　現在行われている検査セットの開発・実用化研究

細胞治療セットⅠ	HBV, HCV, HTLV-1, -2, HIV-1, -2, HHV-1～-8, BKV, JCV, ParvoB19, AdV, Mycoplasma
細胞治療セットⅡ	HBV, HCV, HTLV-1, -2, HIV-1, -2, Mycoplasma
移植セット	HHV-1～-8, BKV, JCV, ParvoB19, AdV
肝炎ウイルスセット	HAV, HBV, HCV, HDV, HEV, GBV, TTV, EBV, CMV
呼吸器ウイルスセット	RSV, MPV, BoV, FluA, S-OIV, FluB, AdV, PIV1, 2, 3, RV, CoV, PeV

MPV：metapneumovirus, S-OIV：novel swine-origin influenza A (H1N1) virus, PIV：parainfluenza virus, RV：rhinovirus, CoV：coronavirus, PeV：parechovirus.

プローブを安定化剤とともに検査プレートあるいは8連ストリップのwellに固相化し，検査にあたっては検体とバッファー，dNTP，増幅酵素の混合液を加えた後に撹拌してプライマー・蛍光プローブを溶解し，PCR反応を開始する．図6bは造血幹細胞移植後のウイルス検査系で，マルチプレックスPCR法により1 wellで3種類のウイルスを測定し，12種類のウイルスを4つのwellで検査することができる．3種類程度のマルチプレックスPCRであれば，組み合わせにもよるが，比較的に高感度は維持されている．陽性ウイルス種は陽性シグナルが出る蛍光色素の違いで識別し，半定量的な解析が可能である．本検査系では，図6cに示すように既知濃度のスタンダードを加えたwellを含む定量用ストリップをあらかじめ用意し，2段階で定量検査を行っている．これは，使用する検体量を少なくし，さらに試薬代の節約と強陽性に隠れた弱陽性項目の見落としをなくすためであり，迅速性が優先される場合には初めから1 wellで1ウイルスの検査を行うシステムとすることも可能である．本検査ストリップの保存安定性を検討したところ，室温で1ヵ月保存しても感度低下が全くみられないことを確認しており，さらなる長期保存後の安定試験を行っている．

固相化プローブ・蛍光プライマーを使用する検査系は，RT-PCR法に応用することが可能であり，現在表2に示すように様々な検査セットの開発・実用化研究を行っている．

おわりに

本節では，網羅的病原微生物検出法開発の経緯と，現在筆者の研究室で実際に稼働している2つの検査法に関して説明した．眼科検体（前房水や硝子体液など）は100μL以下の少量しか得られないことが多く，PCR法により多数の検査項目を同時に検査するためには，キャピラリーを使用したマルチプレックスPCR法が極めて有用である．一方，プライマー・蛍光プローブを固相化した検査プレート・ストリップを用いる方法は，多くの施設に普及しているプレートタイプのリアルタイムPCR機が使用でき，試薬も長期間室温保存でき，検査のセットアップも簡便なため，手軽に多数の微生物検査を同時に行える利点があるが，比較的多くのwellを使用するため，増幅酵素代が嵩むのが欠点である．このような点を踏まえ，筆者らの研究室では検査目的，検体の種類，検査対象項目の種類，などにより検査系を使い分けているとともに，新しい検査セットの開発を継続的に進めている．本節では詳しく触れられなかったが，眼科の感染症検査のようにウイルス検査に加えて他の微生物の検査も同時に行える検査系の開発も順次進めている．今後，誰でも簡便に使用できるように検査試薬をキット化し，本学以外の施設・研究者との共同研究も積極的に進めていく予定である．本検査系の技術移転や技術指導を希望する場合は，下記のアドレスにご連絡いただきたい（清水則夫：nshivir@tmd.ac.jp）．

（清水則夫，渡邊　健）

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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

Accepted 4 April 2010
Published Online First 31 July 2010

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).

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 1.7×10^3 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

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

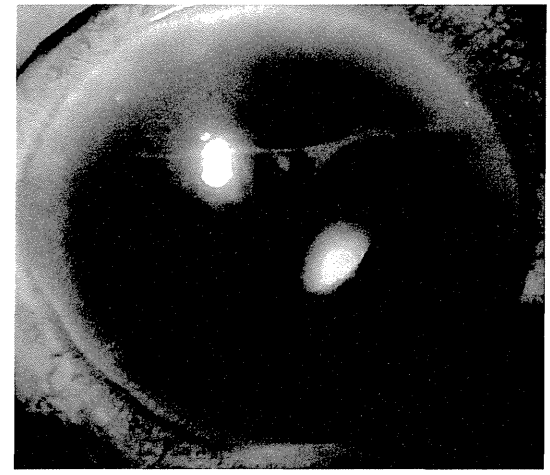

Figure 1 Case 7 (late postoperative endophthalmitis). Slit-lamp photograph in suspected bacterial endophthalmitis. In the right eye, cyclitic membrane, height of the hypopion, and severity of vitritis were seen. In this patient, broad-range quantitative PCR revealed a high copy number of the bacterial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

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 3 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, 1×10^3–1×10^4 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.[13] 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

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.

Acknowledgements Dr Masaru Miyanaga of Miyata Hospital, and Drs Kazuichi Maruyama and Kenji Nagata of the Department of Ophthalmology, Kyoto Prefectural University of Medicine, kindly collected and sent the samples used in this study. We are very grateful for the expert technical assistance of Ms Shizu Inoue. This work was supported by Grants-in-Aid for Scientific Research (C) 20592073 and (B) 19390440 of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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.

Provenance and peer review Not commissioned; externally peer reviewed.

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Sunao Sugita, Norio Shimizu, Ken Watanabe, et al.

Br J Ophthalmol 2011 95: 345-349 originally published online July 31, 2010
doi: 10.1136/bjo.2009.171504

Journal of Pathology
J Pathol 2011; **223:** 496–510
Published online 5 January 2011 in Wiley Online Library
(wileyonlinelibrary.com) DOI: 10.1002/path.2823

ORIGINAL PAPER

Activated oncogenic pathways and therapeutic targets in extranodal nasal-type NK/T cell lymphoma revealed by gene expression profiling

Siok-Bian Ng,[1]* Viknesvaran Selvarajan,[1] Gaofeng Huang,[2] Jianbiao Zhou,[3] Andrew L Feldman,[4] Mark Law,[4] Yok-Lam Kwong,[5] Norio Shimizu,[6] Yoshitoyo Kagami,[7] Katsuyuki Aozasa,[8] Manuel Salto-Tellez[1,3] and Wee-Joo Chng[2,3]*

[1] *Department of Pathology, National University Health System, Singapore*
[2] *Department of Haematology-Oncology, National University Cancer Institute of Singapore, National University Health System, Singapore*
[3] *Cancer Science Institute Singapore, National University of Singapore, Singapore*
[4] *Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, USA*
[5] *Division of Haematology/Oncology and Bone Marrow Transplantation, Queen Mary Hospital, Hong Kong*
[6] *Department of Virology, Tokyo Medical and Dental University, Japan*
[7] *Department of Hematology, Toyota Kosei Hospital, Japan*
[8] *Department of Pathology, Osaka University Graduate School of Medicine, Japan*

**Correspondence to: Dr Siok-Bian Ng, MBBS, FRCPA, Department of Pathology, National University Hospital, 5 Lower Kent Ridge Road, Main Building, Level 3, Singapore 119074. e-mail: patnsb@nus.edu.sg*
**Correspondence to: Dr Wee-Joo Chng, MB ChB, MRCP, FRCPath, Department of Haematology–Oncology, National University Hospital, 5 Lower Kent Ridge Road, Main Building, Level 3, Singapore 119074. e-mail: mdccwj@nus.edu.sg*

Abstract

We performed comprehensive genome-wide gene expression profiling (GEP) of extranodal nasal-type natural killer/T-cell lymphoma (NKTL) using formalin-fixed, paraffin-embedded tissue ($n = 9$) and NK cell lines ($n = 5$) in comparison with normal NK cells, with the objective of understanding the oncogenic pathways involved in the pathogenesis of NKTL and to identify potential therapeutic targets. Pathway and network analysis of genes differentially expressed between NKTL and normal NK cells revealed significant enrichment for cell cycle-related genes and pathways, such as PLK1, CDK1, and Aurora-A. Furthermore, our results demonstrated a pro-proliferative and anti-apoptotic phenotype in NKTL characterized by activation of Myc and nuclear factor kappa B (NF-κB), and deregulation of p53. In corroboration with GEP findings, a significant percentage of NKTLs ($n = 33$) overexpressed c-Myc (45.4%), p53 (87.9%), and NF-κB p50 (67.7%) on immunohistochemistry using a tissue microarray containing 33 NKTL samples. Notably, overexpression of survivin was observed in 97% of cases. Based on our findings, we propose a model of NKTL pathogenesis where deregulation of p53 together with activation of Myc and NF-κB, possibly driven by EBV LMP-1, results in the cumulative up-regulation of survivin. Down-regulation of survivin with Terameprocol (EM-1421, a survivin inhibitor) results in reduced cell viability and increased apoptosis in tumour cells, suggesting that targeting survivin may be a potential novel therapeutic strategy in NKTL.

Keywords: NK/T-cell lymphoma; gene expression profiling; survivin; Myc; NF-κB; p53; paraffin-embedded tissue

Received 9 September 2010; Revised 3 November 2010; Accepted 4 November 2010

No conflicts of interest were declared.

Introduction

Extranodal nasal-type natural killer/T-cell lymphoma (NKTL) is a distinct clinicopathological entity most commonly affecting Asians and Central and South Americans, and characterized by a clonal proliferation of NK or T cells with a cytotoxic phenotype [1]. There is a strong association with Epstein–Barr virus (EBV), which manifests a type II latency pattern [2,3]. EBV is detected in the neoplastic cells in a clonal episomal form, supporting the role of the virus in tumour pathogenesis. There have been few studies investigating the oncogenic mechanisms of NKTL. These reports have identified mutations of genes regulating apoptosis, such as *FAS* and *p53*, which may contribute to the development of this tumour [4–6]. In addition, the expression of P-glycoproteins [7] and absence of granzyme B inhibitor PI9 [8] may account for the poor prognosis of patients with NKTL who were treated with chemotherapy. Like many haematolymphoid malignancies, NKTL is frequently associated with genetic alterations involving loss or gain of genetic material, the