Table Legends

Table 1. Related cell line sets: locus differences

Number of locus differences seen when STR profiles are grouped into related cell line sets. For all of the related cell line sets (n = 369), profiles were compared to a single reference profile, and the maximum number of locus differences arising from that comparison was recorded. Results for individual STR profiles from the combined dataset (n = 1157) are shown in the remaining rows, excluding the reference profiles, which are used for comparison (n = 788). Results are sorted by their relationship to the reference profile – samples from the same cell line, legitimately related samples, misidentified or cross-contaminated cell lines, and those with an unknown relationship.

Table 2. Related cell line sets: match criteria

Effectiveness of the match criteria recommended within the Standard to determine relatedness for cell line samples. For all of the related cell line sets (n = 369), the Masters algorithm (see Methods) was used to compare individual sample profiles to a designated reference profile. The lowest percent match for each set is recorded in the first row of the table. Results for individual STR profiles from the combined dataset (n = 1157) are shown in the remaining rows, excluding the reference profiles, which are used for comparison (n = 788). Results are sorted by their relationship to the reference profile – samples from the same cell line, legitimately related samples, misidentified or cross-contaminated cell lines, and those with an unknown relationship. The columns falling below the 80 % match threshold are highlighted in grey.

Additional Supporting Material for Online Publication

Supplementary Table 1. Cell line sets failing to meet the 80 % match threshold

An Excel spreadsheet lists all of the related cell line sets that fail to meet the 80 % threshold, and the STR profiles that make up each set. The table includes the cell line name; the contributing cell bank (de-identified as to the identity of the cell bank, A-H); the STR profile; and the percent match according to the three match algorithms used.

Supplementary Table 2. Misidentified or cross-contaminated cell lines

An Excel spreadsheet lists all of the STR profiles from known misidentified or cross-contaminated cell lines within the dataset. The table includes the reference profile used for comparison; the cell line name; the contributing cell bank (de-identified as to the identity of the cell bank, A-H); the STR profile; and the percent match according to the three match algorithms used.

Table 1. Related cell line sets: locus differences

Sample or Set Under Consideration	All loci same (0 different)	8/9 loci same (1 locus different)	7/9 loci same (2 loci different)	≤6/9 loci same (≥3 loci different)	TOTAL
Related Cell Line Sets					
TOTAL	189	93	50	37	369
Individual STR Profiles within the Related Cell Line Sets					
STR profiles in each set from the same cell line	153	101	52	46	352
STR profiles in each set from legitimately related cell lines	107	90	56	77	330
STR profiles in each set from misidentified or cross-contaminated cell lines	20	13	14	44	91
STR profiles in each set with an unknown relationship	5	2	5	3	15

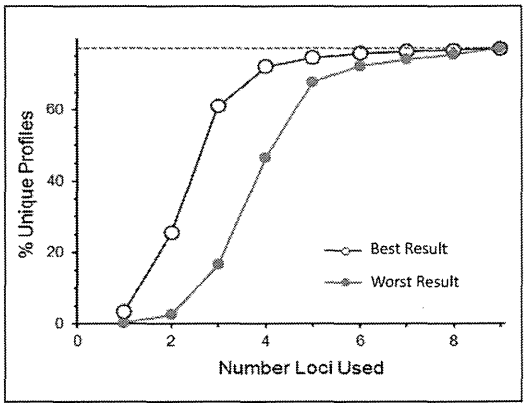
Table 1

Table 2. Related cell line sets: match criteria

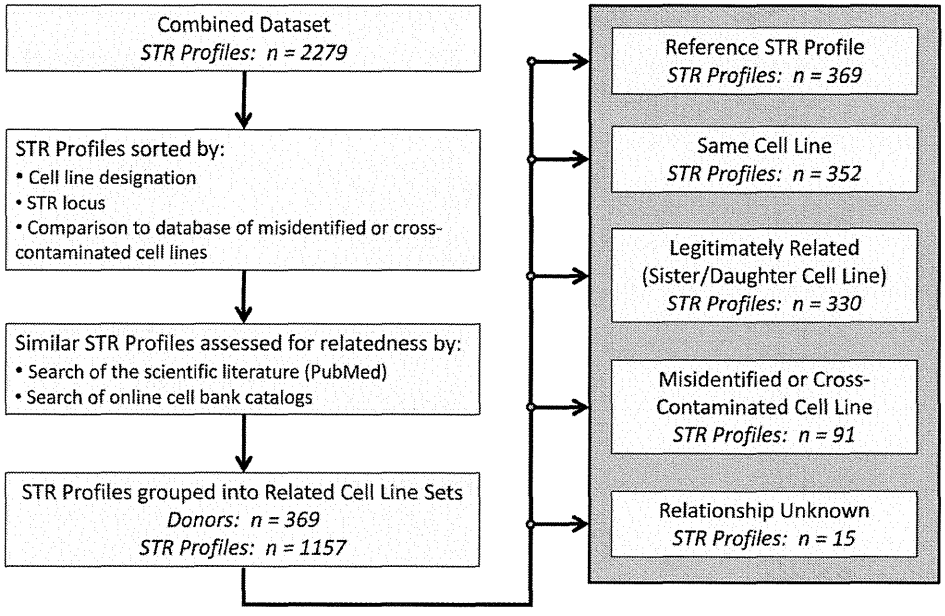
Sample or Set Under Consideration	Lowest match 90 % to 100 %	Lowest match 80 % to 89 %	Lowest match 70 % to 79 %	Lowest match <70 %	TOTAL
Related Cell Line Sets					
TOTAL	327	35	4	3	369
Individual STR Profiles within the Related Cell Line Sets					
STR profiles in each set from the same cell line	324	22	4	2	352
STR profiles in each set from legitimately related cell lines	300	28	1	1	330
STR profiles in each set from misidentified or cross-contaminated cell lines	84	5	1	1	91
STR profiles in each set with an unknown relationship	11	4	0	0	15

Table 2

Number Loci Used	% Unique profiles	
	Best Result	Worst Result
9	77.2	77.2
8	76.8	75.5
7	76.3	74.2
6	75.9	72.3
5	74.6	67.8
4	72.2	46.6
3	61.0	16.6
2	25.4	2.5
1	3.38	0.1



113x47mm (300 x 300 DPI)



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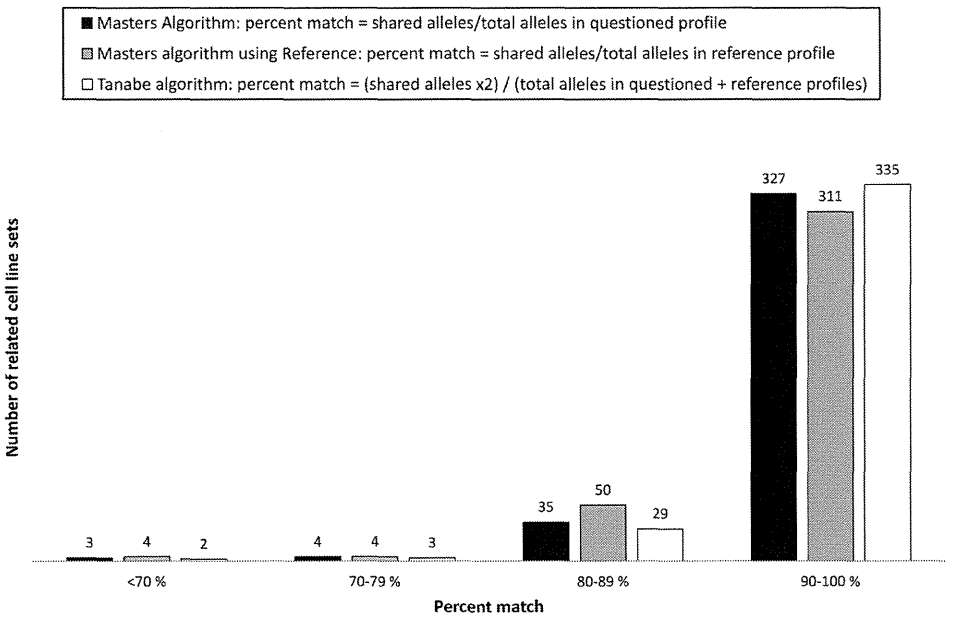

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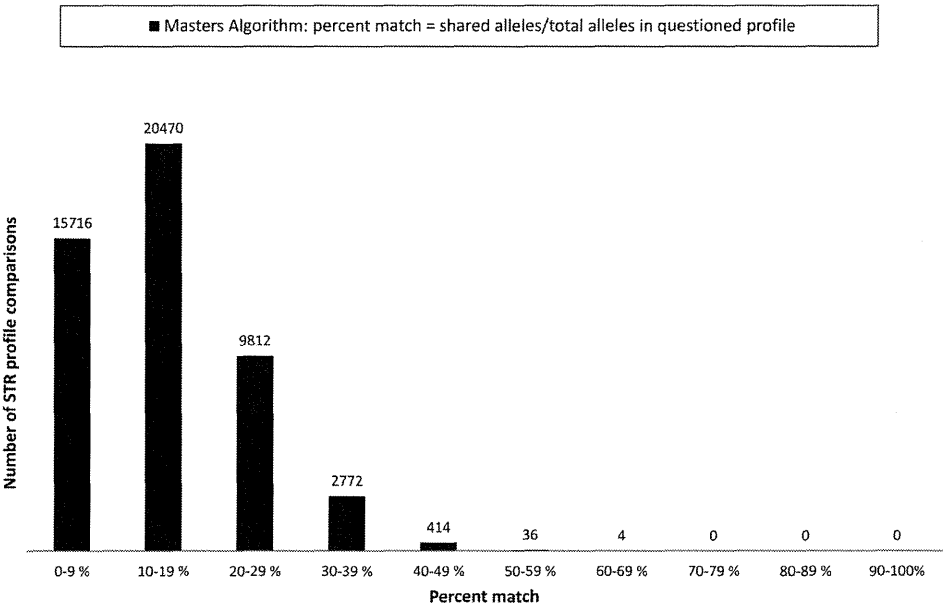

160x101mm (300 x 300 DPI)

Supplementary Table 1. Cell line sets failing to meet the 80% match threshold

This table lists all of the related cell line sets that fail to meet the 80% threshold, and the STR profiles that make up each set.
Data include the cell line name; the contributing cell bank (deidentified, A-H); the STR profile; and the percent match according to the different match algorithms used.
Four cell banks contributed data to this analysis. More than four labels are used (A-H) because some cell banks contributed subsets from their collections that were labelled separately.
Green rows = Reference sample chosen for that set. Shared alleles are in comparison to that reference sample. Red cells = percent match <80%.
In the Relationship column, S = same cell line; D = legitimately related cell line; M = misidentified cell line.
Masters v. Q: percent match = shared alleles/total alleles in the questioned profile.
Masters v. R: percent match = shared alleles/total alleles in the reference profile.
Tanabe algorithm: percent match = (shared alleles x 2)/(total alleles in questioned + reference profiles)

		Locus names									Algorithm calculations and Relationships					
Cell Bank (Code)	Cell name	D5S818	D13S317	D7S820	D16S539	VWA	TH01	AM	TPOX	CSF1PO	Total Alleles	Shared Alleles	Masters v. Q	Masters v. R	Tanabe Algorithm	Relationship
E	CCRF-CEM	12,13	11	9,13	10,13	17,19	6,7	X	7,8	10,13	16					Reference
B	CCRF-CEM	12,13	**11,12**	9,13	10,13	17,19	6,7	X	**8**	**10,11**	16	14	88%	88%	88%	S
C	CCRF-CEM	12,13	**11,12**	**9,12**	10,13	17,19	6,7	X	**8**	**11**	15	12	80%	75%	77%	S
C	CCRF-CEM	12,13	**11,12**	9,13	10,13	17,19	6,7	X	**8**	**10,11**	16	14	88%	88%	88%	S
D	CCRF-CEM	12,13	**10,11,12**	**9,12**	**10,13,14**	**18,19**	6,7	X	**8**	**9,10,11**	19	12	63%	75%	69%	S
A	CCRF-CEM [CCRF CEM]	12,13	**11,12**	9,13	10,13	17,19	6,7	X	**8**	**10,11**	16	14	88%	88%	88%	S
A	CEM-CM3	12,13	**12**	**10,13**	**10,12**	**18,19**	6,7	X	**8**	**11**	14	9	64%	56%	60%	D
E	JURKAT	9	8,11	8,10,11	11	18,19,17	6,9.3	X,Y	8,10	10,11	18					Reference
D	Jurkat	9	**8,11,12**	8,10,11	11	**16,17,18**	6,9.3	X,Y	8,10	**11**	18	16	89%	89%	89%	S
A	Jurkat, Clone E6-1	9	**8,12**	**8,12**	11	**18**	6,9.3	X,Y	8,10	**11,12**	15	12	80%	67%	73%	D
A	J.CaM1.6 (derivative mutant of Jurkat)	9	**8,12**	**8,10**	**10,11**	**18,19**	6,9.3	X,Y	8,10	**11**	16	14	88%	78%	82%	D
A	J.gamma1	9	**8,12**	**8,12**	11	**18,19**	6,9.3	**X**	8,10	**11,12**	15	12	80%	67%	73%	D
D	JM	9	8,11	**8,10**	**10,11**	**16,17,18**	6,9.3	X,Y	**8,9,10**	**9,10,11**	20	16	80%	89%	84%	D
A	A3	9	8,11	**8,10**	11	**17,18**	6,9.3	X,Y	8,10	**11,12**	16	15	94%	83%	88%	D
A	I 2.1	9	8,11	**8,10**	11	**17,18**	6,9.3	X,Y	8,10	**11,12**	16	15	94%	83%	88%	D
A	I 9.2	9	8,11	**8,9.2**	11	**18**	6,9.3	**X**	8,10	**11,12**	14	12	86%	67%	75%	D
A	D1.1	9	8,11	**8,10**	**10,11**	**18,19,20**	6,9.3	**X**	8,10	**11,12**	17	14	82%	78%	80%	D
A	P116	9	**8,12**	**8,11.3**	11	**18,19**	6,9.3	**X**	8,10	**11,12**	15	12	80%	67%	73%	D
E	KCL-22	10,12	8,11	12	12	14	7,9	X	8	12	12					Reference
C	KCL-22	**10,11**	**8,12**	**11,12**	12	14	7,9	X	8	12	13	10	77%	83%	80%	S

Cell Bank (Code)	Cell name	Locus names									Algorithm calculations and Relationships					
		D5S818	D13S317	D7S820	D16S539	VWA	TH01	AM	TPOX	CSF1PO	Total Alleles	Shared Alleles	Masters v. Q	Masters v. R	Tanabe Algorithm	Relationship
E	KYSE-30	11	9	11,12	10,12	16,18	9,9	X	9	10	12					Reference
C	KYSE-30	11	9	**11,11.3**	10,12	**16,18,19**	9,9	X	**8,9**	10	14	11	79%	92%	85%	S
E	MOLT-3	12,13	12,13	8,10,7	11,14	17,18	6,8	X,Y	8	11,12	18					Reference
D	MOLT-3	12,13	**11,12,13**	**7,8,9**	**10,11,13**	17,18	6,8	X,Y	8	**11,12,13**	21	16	76%	89%	82%	S
E	MOLT-4	**11,12**	12,13	**8,10**	11,14	17,18	6,8	X,Y	8	11,12	17	16	94%	89%	91%	D
B	MOLT-4	**12**	12,13	**8,10,11**	11,14	17,18	6,8	X,Y	8	**11,12,13**	18	16	89%	89%	89%	D
C	MOLT-4	**12**	12,13	**8,10,11**	**11,14,15**	17,18	6,8	X,Y	8	**11,12,13**	19	16	84%	89%	86%	D
D	MOLT-4	**12**	12,13	**8,10,11**	**11,14,15**	17,18	6,8	X,Y	8	**11,12,13**	19	16	84%	89%	86%	D
D	MOLT-4F	**11,12,13**	12,13	7,8,10	**11,12,13**	**17,18,19**	6,8	X,Y	8	11,12	21	16	76%	89%	82%	D
E	MT-3	12	10,11	11,12	11,13	14,17	6,7	X	8,9	10,13	16					Reference
C	MT-3	**11**	10,11	**10.3,12**	**9,11**	14,17	**9,9**	**X,Y**	8,9	**10,12**	16	10	63%	63%	63%	S
E	NALM-6	11,12,10	9,12,8	8,10,9	10,11,9	15,16	8,9	X,Y	8,10	12	22					Reference
D	NALM-6	**11,12**	**9,13**	**8,11**	**10,11**	15,16	8,9	X,Y	8,10	**13**	17	14	82%	64%	72%	S
B	SNG-II	6,12	9,12	9,13	12,13	15,16,17	9,9	X	11	11,14	16					Reference
B	RTSG	6,12	9,12	**9,12,13**	**12,13,15**	15,16,17	9,9	X	11	**11,13**	18	15	83%	94%	88%	M
B	RTSG	6,12	**8,9,12**	**9,11,12**	**12,13,14**	15,16,17	9,9	X	11	**11,13**	19	14	74%	88%	80%	M
B	RMUG-L	**6,12,13**	**8,9,11**	**8,9,12**	**12,13,14**	15,16,17	9,9	X	11	**11,13**	20	13	65%	81%	72%	M
C	U937	12	10,12	9,11	12	14,15	6,10	X	8,11	12	14					Reference
D	U937	12	10,12	9,11	12	14,15	**6,9.3**	X	8,11	12	14	13	93%	93%	93%	S
E	U-937	**10,12,13**	10,12	9,11	12	14,15	**6,9.3**	X	8,11	**10,12**	17	13	76%	93%	84%	S
A	U-937	12	10,12	9,11	12	**15**	**9.3**	X	8,11	12	12	11	92%	79%	85%	S
B	U-937	12	10,12	9,11	12	14,15	**6,9.3**	X	8,11	12	14	13	93%	93%	93%	S
C	U937 cl1-14	12	10,12	9,11	12	14,15	**6,9.3**	X	8,11	12	14	13	93%	93%	93%	D
C	U937 d 1-22	12	10,12	9,11	12	14,15	**6,9.3**	X	8,11	12	14	13	93%	93%	93%	D
D	U-937 DE-4	12	10,12	9,11	12	**15**	**6,9.3**	X	8,11	12	13	12	92%	86%	89%	D
E	JOSK-I	12	10,12	9,11	12	14,15	**6,9.3**	X	8,11	12	14	13	93%	93%	93%	M
E	JOSK-M	**13**	10,12	9,11	12	14,15	**6,9.3**	X	8,11	12	14	12	86%	86%	86%	M
A	TUR	12	10,12	9,11	12	**14,16**	**6,9.3**	X	8,11	12	14	12	86%	86%	86%	D

Match criteria for human cell line authentication: Supplementary Table 1

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CLINICAL INVESTIGATION

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

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

Keywords Endophthalmitis · Bacteria · Polymerase chain reaction

Introduction

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

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

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

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

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

Methods

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

Bacterial strains

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

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

DNA extraction

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

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

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

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

Real-time PCR

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

Sensitivity of real-time PCR assay

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

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

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

Results

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

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

Sensitivity of the PCR assay for vitreous samples

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

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

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

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

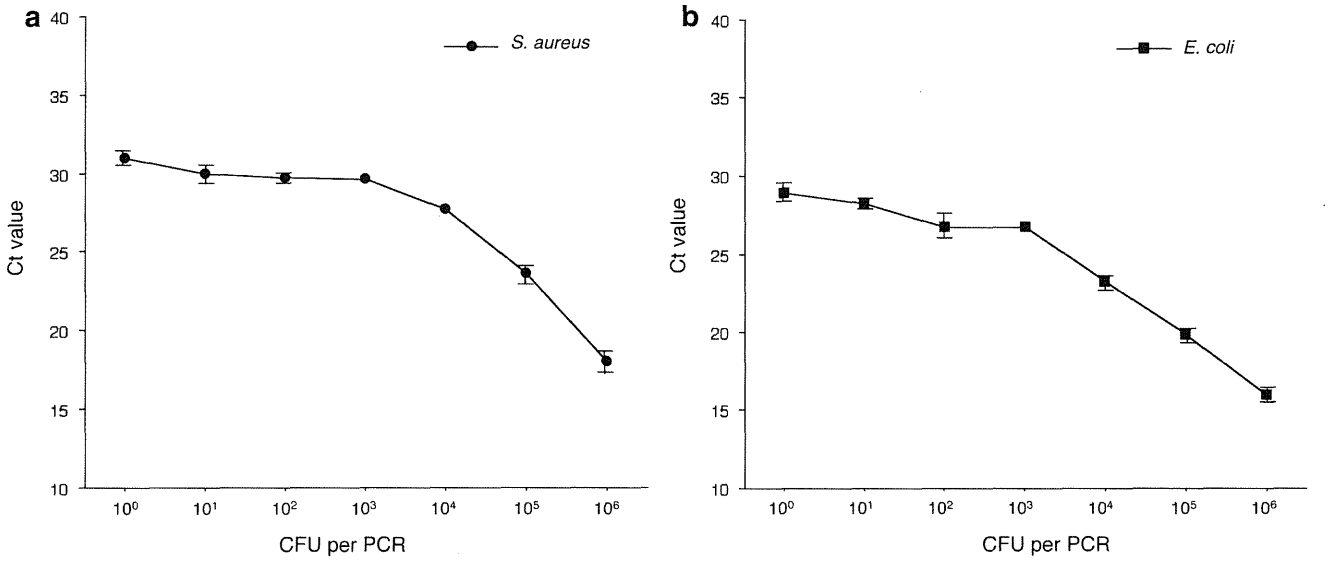

Fig. 1 Analytical detection ranges and sensitivities of a broad-range real-time PCR assay in diluted vitreous samples. **a** Detection of *S. aureus*. **b** Detection of *E. coli*. Results shown are the means and standard deviations of three independent experiments

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

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

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

Discussion

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

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

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

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

MRSA methicillin-resistant *Staphylococcus aureus*

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

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

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

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

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

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

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

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

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

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

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

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INFLAMMATORY DISORDERS

Novel diagnosis of fungal endophthalmitis by broad-range real-time PCR detection of fungal 28S ribosomal DNA

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Abstract
Aim To detect the fungal genome in the ocular fluids of patients with fungal endophthalmitis by using a novel broad-range polymerase chain reaction (PCR) system.
Methods After informed consent was obtained, ocular fluid samples (aqueous humor or vitreous fluids) were collected from 497 patients (76 patients with infectious endophthalmitis including clinically suspected bacterial and fungal endophthalmitis and 421 patients with infectious or non-infectious uveitis). Forty ocular samples from non-infectious patients without ocular inflammation were collected as controls. Fungal ribosomal DNA (28 S rDNA) was measured by a quantitative real-time PCR assay.
Results Fungal 28 S rDNA of the major fungal species, such as *Candida*, *Aspergillus*, and *Cryptococcus*, were detected by novel broad-range real-time PCR examination ($>10^1$ copies/ml). Fungal 28 S rDNA was detected in the ocular fluids of 11 patients with endophthalmitis or uveitis (11/497, 2.2%). All 11 positive samples were detected in the infectious endophthalmitis patients (11/76, 14.5%). These PCR-positive ocular fluids had high copy numbers of fungal 28 S rDNA (range, 1.7×10^3 to 7.9×10^6 copies/ml), which indicated the presence of fungal infection. Of the 11 patients who were PCR positive, further examinations led to a diagnosis of fungal endophthalmitis in ten patients. The fungal 28 S rDNA was detected in one non-infectious case (a false-positive case). In addition, there were two PCR false-negative cases that were clinically suspected of having fungal endophthalmitis.
Conclusions This novel quantitative broad-range PCR of fungal 28 S rDNA is a useful tool for diagnosing endophthalmitis related to fungal infections.

Keywords Polymerase chain reaction · Fungi · Ocular fluids · Endophthalmitis

Introduction

Fungal endophthalmitis can be caused by endogenous infections. These infections occur in patients who have systemic disorders (e.g., diabetes or malignancy), patients who use systemic drugs (e.g., broad-spectrum antibiotics, chemotherapeutic agents, or steroids), and patients who have intravascular catheters. In addition, fungal endophthalmitis can be caused by exogenous infections that arise from trauma or intraocular surgery. The clinical findings in some ocular infectious diseases caused by fungal species are quite diverse, with the exception of *Candida* infection. *Candida* infection in the eye is always characterized as endogenous endophthalmitis with fungal ball vitreous opacities. Moreover, fungal infections have been widely associated with various ocular disorders including endophthalmitis. Because of this diversity, it is often difficult to diagnose ocular fungal infections. Polymerase chain reaction (PCR) has been used to provide evidence of fungal involvement in suspected cases of intraocular infections. Previous studies have used PCR to demonstrate the presence of

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fungal DNA in the ocular fluids of patients with infectious endophthalmitis [1–3].

PCR-based methods make it possible to establish a diagnosis in less time than is required by standard cultures [4–6]. Moreover, studies have found that fungal cultures are negative in half of PCR-positive cases [1–3, 7]. The sensitivity of conventional culture techniques is not high, and these cultures take a long time due to their slow growth. Thus, the use of broad-range real-time PCR to analyze ocular samples may be a better way to obtain a rapid diagnosis in patients with unknown intraocular infectious diseases.

For the diagnosis of infectious endophthalmitis, broad-range real-time PCR for fungi is now available [1–3, 7]. To detect many types of fungal DNA, primers and probes for conserved regions in fungal sequences are used. We previously designed pan-fungal primers and probes that were complementary to the 18 S rRNA sequences present in the *Candida* and *Aspergillus* species, and we reported the efficacy of the technique for diagnosis [7]. This PCR technique detected all species of *Candida* and *Aspergillus* DNA. Although there were many advantages to using this PCR technique to diagnose fungal infection, there was one disadvantage. Although the fungal 18 S broad-range PCR detected *Candida* and *Aspergillus* DNA, it cannot detect other types of fungal DNA. Recently, a novel broad-range real-time PCR technique was developed for the rapid detection of human pathogenic fungi [8]. The assay targeted a part of the 28 S large subunit rRNA genes (28 S rDNA). Therefore, we prepared a new assay that targets a part of the 28 S rDNA found in species such as *Candida*, *Aspergillus*, *Cryptococcus*, *Trichophyton*, *Mucor*, *Penicillium*, and *Pichia*.

In the present study, we attempted to develop a novel fungal PCR examination that uses 28 S rDNA primers and the corresponding probes for the diagnosis of endophthalmitis related to fungal infection.

Materials and methods

Subjects

Based upon medical history and clinical observations, 497 patients (260 men and 237 women) were consecutively enrolled in a prospective study that was conducted at the Tokyo Medical and Dental University Hospital. The patient group consisted of patients with infectious endophthalmitis including clinically suspected bacterial and fungal endophthalmitis (n=76) and patients with infectious or non-infectious uveitis (n=421). The average patient age (±SD) was 60 (±16) years. After obtaining informed consent, samples of aqueous humor and vitreous fluids were collected from all patients.

In addition to the patient group, we also analyzed samples from a control group in which no patients had any type of ocular inflammation. The control patients were enrolled in this prospective study in 2009. Forty samples (20 aqueous humor samples and 20 vitreous fluid samples) were collected from the 40 control patients. The control group consisted of patients who had age-related cataract (n=20), macular edema (n=14), retinal detachment (n=4), idiopathic macular hole (n=1), and idiopathic epiretinal membrane (n=1).

For aseptic ocular sampling, the following procedures were performed in all subjects, as described in our previous reports [7, 9]. A 0.1-ml aliquot of aqueous humor was collected aseptically in a syringe with a 30-G needle. Half of the sample was then transferred into a pre-sterilized microfuge tube and used for PCR. In patients who were undergoing vitreous surgery, uncontaminated non-diluted vitreous fluid samples (0.5–1.0 ml) were collected during the diagnostic pars plana vitrectomy [7, 9]. Topical antibiotics were used in almost all patients before collecting samples, but oral antibiotics were not used.

The research followed the tenets of the Declaration of Helsinki, and all study protocols were approved by the Institutional Ethics Committee of Tokyo Medical and Dental University. The clinical trial was registered, and the information is available at www.umin.ac.jp/ctr/index/htm with study number R000002708. The study was started in July 2009 and was terminated in February 2011.

Quantitative polymerase chain reaction

DNA was extracted from the samples using a DNA Mini Kit (Qiagen, Valencia, CA, USA) installed on a Robotic workstation that was set for automated purification of nucleic acids (BioRobot EZ1 Advanced, Qiagen). The real-time PCR was performed by using an Amplitaq Gold and Light Cycler 480 II (Roche, Basel, Switzerland). Primers and probes of fungal 28 S rDNA are described elsewhere [8]. The sense primer was 5'-gcatatcaataagcggaggaaaag-3', and the antisense primer was 5'-ttagctttagatgRaRtttaccacc-3'. The probe (Dual-Labeled probe, Integrated DNA Technologies, Coralville, IA, USA) was 5'-FAM- cggcgagtgaagcgg-SaaRagctc–iowaBK-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 0 s and 60 °C for 20 s. For PCR assay sensitivity, PCR fragments were amplified from the DNA of *C. albicans* (strain: ATCC 60193), *A. flavas* (strain: ATCC 22546), and *C. neoformans* (strain: ATCC 14116). The PCR results were obtained within 3 h after sample collection.

Amplification of the human β-globulin gene served as an internal positive extraction and amplification control. Fungal copy number values of more than 100 copies/ml in the sample were considered to be significant.

Results

Sensitivity of broad-range real-time PCR assay for fungal 28 S rDNA

To confirm the broad-range real-time PCR assay sensitivity, PCR fragments were amplified from the DNA of *Candida*, *Aspergillus*, and *Cryptococcus* species. The detection limit and standard range of the TaqMan real-time PCR were determined by using serial tenfold dilutions of linearized plasmid. The PCR results for the prepared samples showed that *C. albicans* DNA was detected at concentrations between 10^2 and 10^5 copies/ml (Fig. 1A). In addition, *Aspergillus* (Fig. 1B) and *Cryptococcus* DNA (Fig. 1C) were also detected at concentrations between 10^2 and 10^5 copies/ml. The best sensitivity for detecting *Candida*, *Aspergillus*, or *Cryptococcus* DNA was at a concentration of 10^1 copies/ml. No DNA was detected in the negative control (nuclease-free water).

Detection of fungal 28 S rDNA in suspected fungal endophthalmitis patients

The PCR results indicated that fungal 28 S rDNA was positive in 11 samples of ocular fluid from the endophthalmitis or uveitis patients (11/497, 2.2%). All 11 positive samples were detected in the infectious endophthalmitis patients (11/76, 14.5%). A representative PCR result in a case of endogenous endophthalmitis related to *Aspergillus* infection is shown in Fig. 2.

The PCR-positive patients had high copy numbers of fungal 28 S rDNA ranging from 1.7×10^3 to 7.9×10^6 copies/ml, which indicated the presence of fungal infection. Further examinations revealed that 10 of the 11 PCR-positive patients had fungal endophthalmitis; seven patients were diagnosed with endogenous endophthalmitis (3 *Candida,* 1 *Aspergillus*, 1 *Cryptococcus*, and 2 unknown), and three patients were diagnosed with late postoperative endophthalmitis (2 *Candida* and 1 unknown) (Table 1). Fungal 28 S rDNA was detected in only one non-infectious case (case 455 in Table 1). This PCR false-positive case had primary intraocular lymphoma that was diagnosed by monoclonal detection of B-cell IgH rearrangement by PCR, high amounts of IL-10 by ELISA, and detection of typical lymphoma cells (Class V) in the vitreous sample. Thus, fungal 28 S rDNA was detected in ocular samples from 10 patients with fungal endophthalmitis and one patient with non-infectious primary intraocular lymphoma.

However, two of the PCR-negative patients were clinically suspected to have fungal endophthalmitis (cases 24 and 461 in Table 1). PCR did not detect the fungal genome in the aqueous humor of these patients (<100 copies). *C. albicans* was detected in blood samples from case 461. Case 24 was a patient with endogenous endophthalmitis, and his blood tests were positive for β-D-glucan. Systemic antimycotic and topical antimycotic therapies were effective in the treatment of these two patients with false-negative results.

In conventional fungal cultures of ocular fluids, six (60%) of the 10 PCR-positive samples from fungal endophthalmitis patients were positive, and four samples were negative (Table 1). In addition, patients with fungicemia (cases 24, 179, 231, 326, 359, 461, and 490) had already begun therapy with antimycotic agents before PCR examinations (Table 2). Among these seven patients, six patients received intravenous hyperalimentation. On the other hand, patients without clinically apparent fungicemia (cases 30, 77, 161, and 355) initiated antimycotic drug therapy after receiving positive PCR results (Table 2).

The diagnostic parameters of sensitivity, specificity, positive predictive value, and negative predictive value of the PCR examinations for the diagnosis of fungal endophthalmitis were calculated to be 0.833, 0.998, 0.909, and 0.996, respectively.

Discussion

The broad-range real-time PCR assay amplified fungal 28 S rDNA in the ocular fluids of patients with clinically suspected fungal endophthalmitis. The 28 S rDNA of major fungal species for endophthalmitis, such as *Candida*, *Aspergillus*, and *Cryptococcus*, were detected by a novel broad-range real-time PCR examination (>10^1 copies/ml). Our fungal endophthalmitis patients were all immunocompetent, but almost all patients were older than 60 years of age, with the exception of two patients (cases 268 and 490). The PCR examination was negative for fungal DNA in cases of ocular inflammation caused by bacterial endophthalmitis or uveitis. In addition, fungal DNA was not detected in any of the 40 control patients without ocular inflammation.

Broad-range PCR for the 28 S rRNA sequence proved to be a reliable tool for the diagnosis of fungal endophthalmitis. Moreover, real-time quantitative PCR can be used to determine whether or not the fungus is related to the endophthalmitis. By using this PCR system, we were able to rapidly diagnose various types of fungal endophthalmitis in a few