(15) Schweppe RE, Klopper JP, Korch C, Pugazhenthi U, Benezra M, Knauf JA, Fagin JA, Marlow LA, Copland JA, Smallridge RC, Haugen BR. Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. *J Clin Endocrinol Metab* 2008;**93**:4331-41.

(16) Lorenzi PL, Reinhold WC, Varma S, Hutchinson AA, Pommier Y, Chanock SJ, Weinstein JN. DNA fingerprinting of the NCI-60 cell line panel. *Mol Cancer Ther* 2009;**8**:713-24.

(17) Brenner JC, Graham MP, Kumar B, Saunders LM, Kupfer R, Lyons RH, Bradford CR, Carey TE. Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. *Head Neck* 2010;**32**:417-26.

(18) Zhao M, Sano D, Pickering CR, Jasser SA, Henderson YC, Clayman GL, Sturgis EM, Ow TJ, Lotan R, Carey TE, Sacks PG, Grandis JR, et al. Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites. *Clin Cancer Res* 2011;**17**:7248-64.

(19) Korch C, Spillman MA, Jackson TA, Jacobsen BM, Murphy SK, Lessey BA, Jordan VC, Bradford AP. DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. *Gynecol Oncol* Epub 2012.

(20) National Institute of Standards and Technology. Identification of Human Cell Lines Project. *Fed Regist* 2012;**77**:5489-91.

(21) O'Donoghue LE, Rivest JP, Duval DL. Polymerase chain reaction-based species verification and microsatellite analysis for canine cell line validation. *J Vet Diagn Invest* 2011;**23**:780-5.

(22) Almeida JL, Hill CR, Cole KD. Authentication of African green monkey cell lines using human short tandem repeat markers. *BMC Biotechnol* 2011;**11**:102.

(23) Poetsch M, Petersmann A, Woenckhaus C, Protzel C, Dittberner T, Lignitz E, Kleist B. Evaluation of allelic alterations in short tandem repeats in different kinds of solid tumors--possible pitfalls in forensic casework. *Forensic Sci Int* 2004;**145**:1-6.

(24) Vauhkonen H, Hedman M, Vauhkonen M, Kataja M, Sipponen P, Sajantila A. Evaluation of gastrointestinal cancer tissues as a source of genetic information for forensic investigations by using STRs. *Forensic Sci Int* 2004;**139**:159-67.

(25) MacLeod RA, Dirks WG, Reid YA, Hay RJ, Drexler HG. Identity of original and late passage Dami megakaryocytes with HEL erythroleukemia cells shown by combined cytogenetics and DNA fingerprinting. *Leukemia* 1997;**11**:2032-8.

(26) Chiong E, Dadbin A, Harris LD, Sabichi AL, Grossman HB. The use of short tandem repeat profiling to characterize human bladder cancer cell lines. *J Urol* 2009;**181**:2737-48.

(27) Hane B, Tummler M, Jager K, Schleithoff L, Janssen JW, Drexler HG. Differences in DNA fingerprints of continuous leukemia-lymphoma cell lines from different sources. *Leukemia* 1992;**6**:1129-33.

(28) Parson W, Kirchebner R, Muhlmann R, Renner K, Kofler A, Schmidt S, Kofler R. Cancer cell line identification by short tandem repeat profiling: power and limitations. *FASEB J* 2005;**19**:434-6.

(29) Drexler HG, Dirks WG, Matsuo Y, MacLeod RA. False leukemia-lymphoma cell lines: an update on over 500 cell lines. *Leukemia* 2003;**17**:416-26.

(30) Tanabe H, Takada Y, Minegishi D, Kurematsu M, Msui T, Mizusawa H. Cell line individualization by STR multiplex system in the cell bank found cross-contamination between ECV304 and EJ-1/T24. *Tiss Cult Res Commun* 1999;**18**:329-38.

(31) Bady P, Diserens AC, Castella V, Kalt S, Heinimann K, Hamou MF, Delorenzi M, Hegi ME. DNA fingerprinting of glioma cell lines and considerations on similarity measurements. *Neuro Oncol* 2012;**14**:701-11.

(32) Eltonsy N, Gabisi V, Li X, Russe KB, Mills GB, Stemke-Hale K. Detection algorithm for the validation of human cell lines. *Int J Cancer* 2012;**131**:E1024-E1030.

(33) Lynch M. The similarity index and DNA fingerprinting. *Mol Biol Evol* 1990;**7**:478-84.

(34) Sorensen T. A method of establishing groups of equal amplitude in plant sociology based on similarity of species and its application to analyses of the vegetation on Danish commons. *Biol Skr* 1948;**5**:1-34.

(35) Kataoka E, Honma M, Ohnishi K, Sofuni T, Mizusawa H. Application of highly polymorphic DNA markers to the identification of HeLa cell sublines. *In Vitro Cell Dev Biol* 1992;**28A**:553-6.

(36) Inoue K, Kohno T, Takakura S, Hayashi Y, Mizoguchi H, Yokota J. Frequent microsatellite instability and BAX mutations in T cell acute lymphoblastic leukemia cell lines. *Leuk Res* 2000;**24**:255-62.

(37) Takeuchi S, Takeuchi N, Fermin AC, Taguchi H, Koeffler HP. Frameshift mutations in caspase-5 and other target genes in leukemia and lymphoma cell lines having microsatellite instability. *Leuk Res* 2003;**27**:359-61.

(38) Seitz S, Wassmuth P, Plaschke J, Schackert HK, Karsten U, Santibanez-Koref MF, Schlag PM, Scherneck S. Identification of microsatellite instability and mismatch repair gene mutations in breast cancer cell lines. *Genes Chromosomes Cancer* 2003;**37**:29-35.

(39) Wu W, He Q, Li X, Zhang X, Lu A, Ge R, Zhen H, Chang AE, Li Q, Shen L. Long-term cultured human neural stem cells undergo spontaneous transformation to tumor-initiating cells. *Int J Biol Sci* 2011;**7**:892-901.

(40) Torsvik A, Rosland GV, Bjerkvig R. Spontaneous transformation of stem cells in vitro and the issue of cross-contamination. *Int J Biol Sci* 2012;**8**:1051-2.

(41) Micka KA, Amiott EA, Hockenberry TL, Sprecher CJ, Lins AM, Rabbach DR, Taylor JA, Bacher JW, Glidewell DE, Gibson SD, Crouse CA, Schumm JW.

TWGDAM validation of a nine-locus and a four-locus fluorescent STR multiplex system. *J Forensic Sci* 1999;**44**:1243-57.

(42) Hill CR, Duewer DL, Kline MC, Sprecher CJ, McLaren RS, Rabbach DR, Krenke BE, Ensenberger MG, Fulmer PM, Storts DR, Butler JM. Concordance and population studies along with stutter and peak height ratio analysis for the PowerPlex (R) ESX 17 and ESI 17 Systems. *Forensic Sci Int Genet* 2011;**5**:269-75.

(43) Bacher JW, Flanagan LA, Smalley RL, Nassif NA, Burgart LJ, Halberg RB, Megid WM, Thibodeau SN. Development of a fluorescent multiplex assay for detection of MSI-High tumors. *Dis Markers* 2004;**20**:237-50.

(44) Castro F, Dirks WG, Fahnrich S, Hotz-Wagenblatt A, Pawlita M, Schmitt M. High-throughput SNP-based authentication of human cell lines. *Int J Cancer* Epub 2012.

(45) Daemer RJ, Feinstone SM, Alexander JJ, Tully JG, London WT, Wong DC, Purcell RH. PLC/PRF/5 (Alexander) hepatoma cell line: further characterization and studies of infectivity. *Infect Immun* 1980;**30**:607-11.

(46) Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J, Kwei KA, Hernandez-Boussard T, Wang P, Gazdar AF, Minna JD, Pollack JR. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One* 2009;**4**:e6146.

(47) Nichols AC, Yoo J, Palma DA, Fung K, Franklin JH, Koropatnick J, Mymryk JS, Batada NN, Barrett JW. Frequent Mutations in TP53 and CDKN2A Found by Next-Generation Sequencing of Head and Neck Cancer Cell Lines. *Arch Otolaryngol Head Neck Surg* 2012;**138**:732-9.

Figure Legends

Figure 1. Discrimination between STR profiles based on the number of loci used

The combined dataset (n = 2279) comprised results from eight STR loci (D5S818, D13S317, D7S820, D16S539, vWA, TH01, TPOX and CSF1PO), with the addition of amelogenin for gender determination. To assess discrimination, the number of unique STR profiles existing within the combined dataset with these nine loci was calculated and expressed as a percentage of the total number of STR profiles in the combined dataset. The number of loci was then progressively reduced from nine to one and the number of unique STR profiles calculated for each possible locus combination (512 combinations). The "best result" (highest) and "worst result" (lowest) are recorded for each locus number.

Figure 2. Grouping STR profiles into related cell line sets

The combined dataset of STR profiles was sorted into related cell line sets using the process shown. The process is described further in the Methods section.

Figure 3. Comparison of match algorithms

Percent match results obtained when comparing related cell line sets using different algorithms. Samples are compared using the Masters algorithm, using the total number of alleles from the questioned profile in the calculation; the Masters algorithm, using the total number of alleles from the reference profile in the calculation; and the Tanabe algorithm. The lowest percent match result for each set is recorded here.

Figure 4. Comparison of unrelated cell lines

Percent match results obtained when comparing unrelated cell line samples. A subset of 223 validated samples, with all related samples removed, was used. Each sample was compared to all of the others in the subset, resulting in a total of 49224 unrelated results.

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.

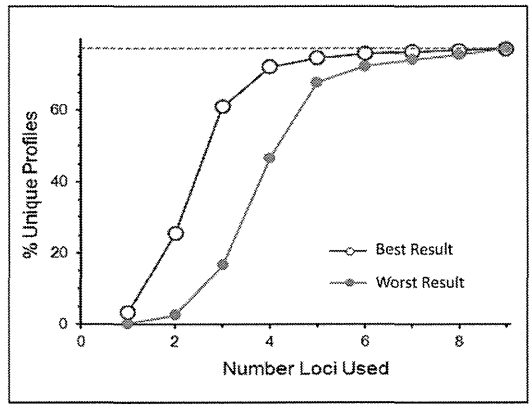
Table 1. Related cell line sets: locus differences

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

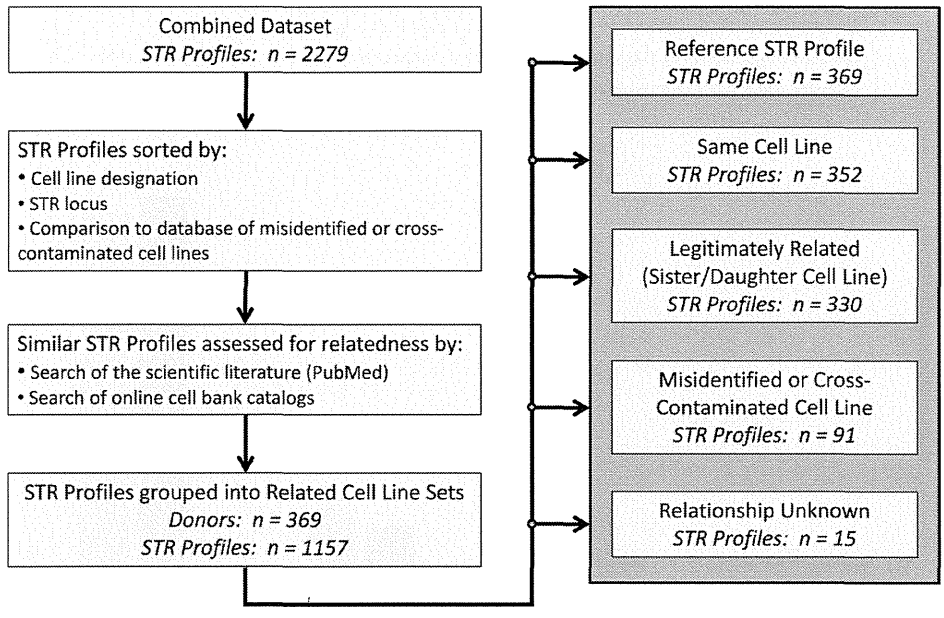
Table 2. Related cell line sets: match criteria

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

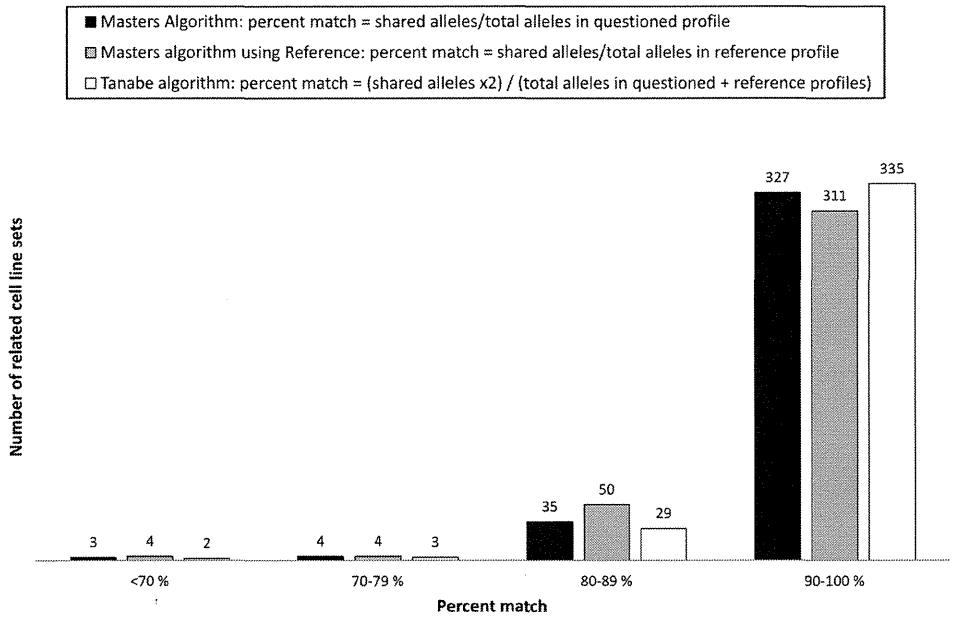
Number Loci Used	% Unique profiles	
	Best Result	Worst Result
9	77.2	77.2
8	76.8	75.5
7	76.3	74.2
6	75.9	72.3
5	74.6	67.8
4	72.2	46.6
3	61.0	16.6
2	25.4	2.5
1	3.38	0.1



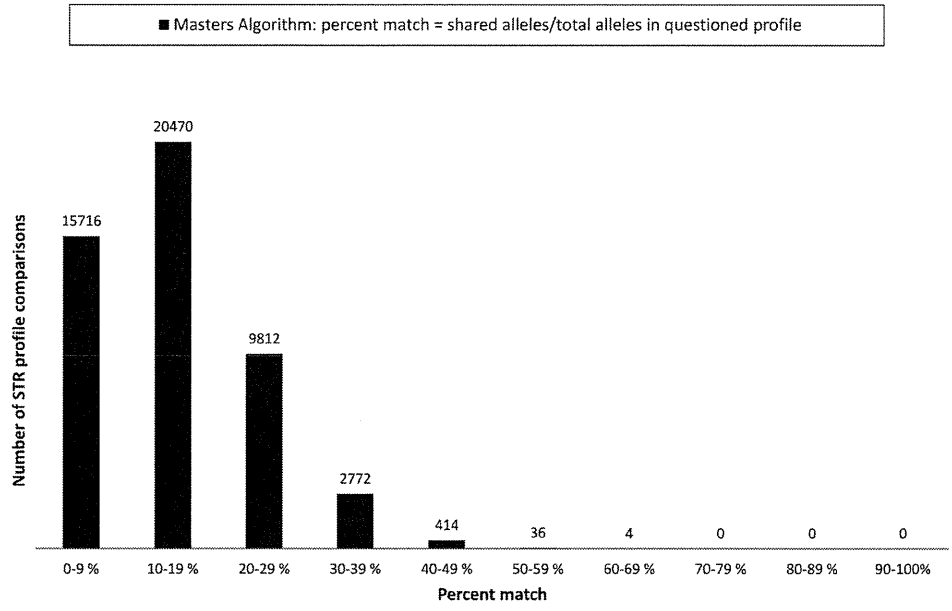
113x47mm (300 x 300 DPI)



164x104mm (300 x 300 DPI)



162x105mm (300 x 300 DPI)



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

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

CLINICAL INVESTIGATION

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

Manabu Ogawa · Sunao Sugita · Norio Shimizu · Ken Watanabe · Ichiro Nakagawa · Manabu Mochizuki

Received: 18 January 2012 / Accepted: 5 July 2012

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

M. Ogawa · S. Sugita · M. Mochizuki
Department of Ophthalmology and Visual Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

S. Sugita (✉)
Laboratory for Retinal Regeneration, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan
e-mail: sunaoph@cdb.riken.jp

N. Shimizu · K. Watanabe
Division of Medical Science, Department of Virology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

I. Nakagawa
Department of Section of Bacterial Pathogenesis, Graduate School of Medicine and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

Introduction

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

Published online: 31 August 2012

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

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

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

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

Methods

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

Bacterial strains

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

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

DNA extraction

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

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

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

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

Real-time PCR

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

Sensitivity of real-time PCR assay

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

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

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

Results

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

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

Sensitivity of the PCR assay for vitreous samples

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

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

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

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

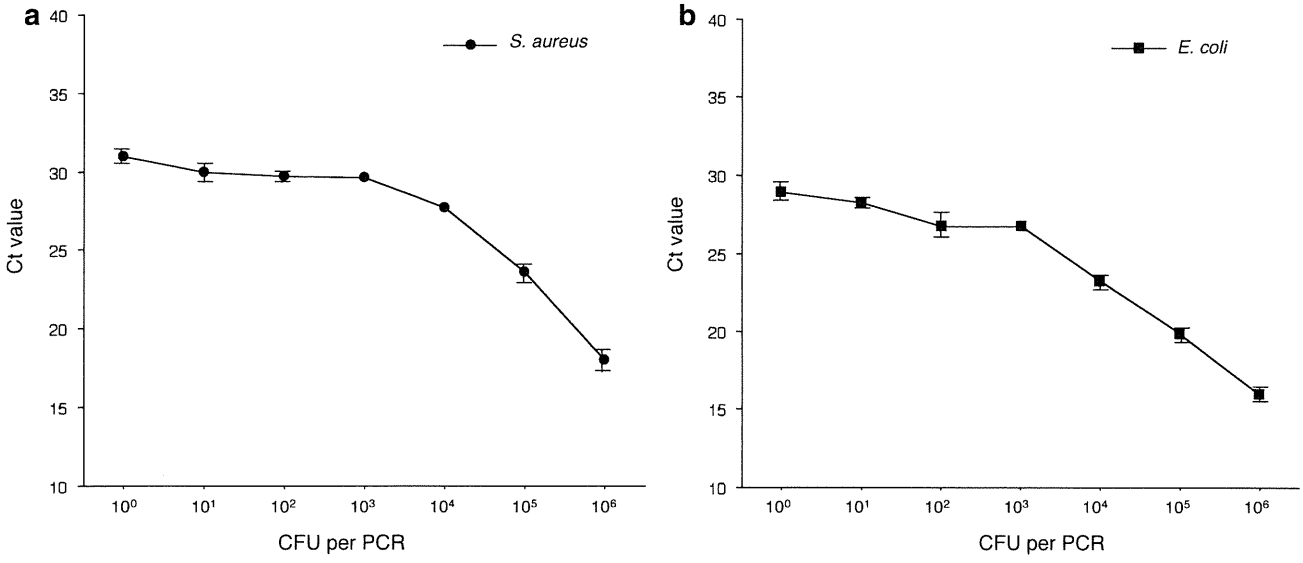


Fig. 1 Analytical detection ranges and sensitivities of a broad-range real-time PCR assay in diluted vitreous samples. **a** Detection of *S. aureus*. **b** Detection of *E. coli*. Results shown are the means and standard deviations of three independent experiments

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

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

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

Discussion

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

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

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

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

MRSA methicillin-resistant *Staphylococcus aureus*

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

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

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

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

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

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

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

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

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

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

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

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

References

1. Brun-Buisson C, Fartoukh M, Lechapt E, Honoré S, Zahar JR, Cerf C, et al. Contribution of blinded, protected quantitative specimens to the diagnostic and therapeutic management of ventilator-associated pneumonia. Chest. 2005;128:533–44.
2. Therese KL, Anand AR, Madhavan HN. Polymerase chain reaction in the diagnosis of bacterial endophthalmitis. Br J Ophthalmol. 1998;82:1078–82.
3. Chiquet C, Cornut PL, Benito Y, Thuret G, Maurin M, Lafontaine PO, et al. Eubacterial PCR for bacterial detection and identification in 100 acute postcataract surgery endophthalmitis. Invest Ophthalmol Vis Sci. 2008;49:1971–8.
4. Sugita S, Shimizu N, Watanabe K, Katayama M, Horie S, Ogawa M, et al. Diagnosis of bacterial endophthalmitis by broad-range quantitative PCR. Br J Ophthalmol. 2011;95:345–9.
5. 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.
6. Zucol F, Ammann RA, Berger C, Aebi C, Altwegg M, Niggli FK, et al. Real-time quantitative broad-range PCR assay for detection of the 16S rRNA gene followed by sequencing for species identification. J Clin Microbiol. 2006;44:2750–9.
7. Usui N, Uno T, Oki K, Oshika T, Ohashi Y, Ogura Y, et al. Nationwide surveillance of postoperative endophthalmitis related to cataract surgery. Jpn J Ophthalmic Surg. 2006;19:73–9.
8. Aaberg TM Jr, Flynn HW Jr, Schiffman J, Newton J. Nosocomial acute-onset postoperative endophthalmitis survey. A 10-year review of incidence and outcomes. Ophthalmology. 1998;105:1004–10.
9. Vafidis G. Propionibacterium acnes endophthalmitis. Br J Ophthalmol. 1991;75:706.
10. Boldt HC, Pulido JS, Blodi CF, Folk JC. Rural endophthalmitis. Ophthalmology. 1989;96:1722–6.
11. Jackson TL, Eykyn SJ, Graham EM, Stanford MR. Endogenous bacterial endophthalmitis: a 17-year prospective series and review of 267 reported cases. Surv Ophthalmol. 2003;48:403–23.
12. Chen YJ, Kuo HK, Wu PC, Kuo ML, Tsai HH, Liu CC, et al. A 10-year comparison of endogenous endophthalmitis outcomes: an east Asian experience with *Klebsiella pneumoniae* infection. Retina. 2004;24:383–90.