Laboratory diagnosis of endophthalmitis: Comparison of microbiology and molecular methods in the European Society of Cataract & Refractive Surgeons multicenter study and susceptibility testing

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PURPOSE: To investigate and compare the use of molecular biology with the use of traditional Gram stain and organism culture for the laboratory diagnosis of postoperative endophthalmitis.

SETTING: Twenty-four ophthalmology units together with 9 microbiology laboratories and 2 European reference molecular biology laboratories.

METHODS: A prospective randomized partially masked multicenter cataract surgery study recruited 16 603 patients. This resulted in 29 cases of presumed postoperative endophthalmitis. Gram stain and culture were performed in the local laboratory according to agreed protocols. Samples of aqueous and/or vitreous were transported to the first referenced molecular biology laboratory (Regensburg, Germany) for polymerase chain reaction (PCR) testing, and an extracted aliquot of DNA was then referred to the second laboratory (Alicante, Spain) for PCR.

RESULTS: Of the 29 who presented with presumed postoperative endophthalmitis, 20 were classified as proven infective endophthalmitis with positive Gram stain, culture, or PCR. Fourteen patients were culture-positive; all but 1 of these was also positive by PCR. Six patients were positive by PCR but negative by Gram stain or culture. Nine patients were negative by both microbiology and PCR testing.

CONCLUSIONS: Use of molecular biology technique increased the laboratory rate of identifying the pathogen by 20%, confirming the technique is very useful for the endophthalmitis specimen. Samples of both aqueous and vitreous should be collected and stored at −20°C for PCR at the time of the diagnostic taps.

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Cataract surgery is one of the most prevalent surgical procedures. Endophthalmitis is an infrequent but vision-threatening complication. The incidence of lost vision due to postoperative (infection) endophthalmitis is increasing in Europe due to an aging population requiring cataract surgery. Endophthalmitis is an inflammatory reaction occurring as a result of intraocular colonization by bacteria. The occurrence, severity, and clinical course of endophthalmitis depend on the route of infection, the virulence and number of inoculated pathogens, and other factors. Coagulase-negative staphylococci account for 33% to 77%, *Staphylococcus aureus* for 10% to 21%, and β-hemolytic streptococci for 9% to 19%.[1–3]

The European Society of Cataract & Refractive Surgeons (ESCRS) recognized the need for a multicenter study to evaluate whether the incidence of endophthalmitis could be significantly reduced by the use of antimicrobial prophylaxis. The purpose of the study was to identify risk factors and describe the effects of antibiotic prophylaxis on the incidence of postoperative endophthalmitis after cataract surgery. Further details on the rationale of this study have been published.[4] The formal registration of the study and its

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protocol can be found at www.clinicaltrials.gov (Accessed June 16, 2008). The members of the ESCRS and Endophthalmitis Study Group are listed in the Appendix.

A preliminary report of the principal findings was published in March 2006[5] and a final statistical report in June 2007.[6] These findings showed that the use of intracameral cefuroxime administered at the time of surgery significantly reduced the risk of cataract patients developing endophthalmitis by a factor of 5. In practice, treatment effects were so marked that fewer than 17 000 patients were sufficient for valid conclusions to be obtained.

The study was planned as a factorial design to test for the effects of 2 prophylactic interventions: (1) cefuroxime injected into the anterior chamber at the end of surgery as 1 mg in 0.1 mL normal saline (0.9%) and (2) levofloxacin 0.5% administered 1 drop 1 hour before surgery, 1 drop 30 minutes before surgery, and 3 drops at 5-minute intervals immediately after surgery.

Patients were randomly assigned to 1 of 4 treatment groups of approximately equal sizes. Group A received minimal prophylaxis of povidone–iodine and neither of the 2 study treatments, Group B received the intracameral cefuroxime treatment only; Group C, the topical levofloxacin treatment only; and Group D, both intracameral cefuroxime and topical levofloxacin treatments. The levofloxacin treatment was masked with patients receiving placebo vehicle or antibiotic drops from bottles supplied as part of the study. The use of cefuroxime was not masked; surgeons were requested to give the intracameral injection at the time of surgery to patients who had been randomly allocated by the operating room database computer to Groups B and D.

All patients received povidone–iodine 5% (Betadine) as 1 drop into the conjunctival sac and onto the cornea for a minimum of 3 minutes before surgery. In addition, all patients were given levofloxacin 0.5% eyedrops (Oftaquix) for 6 days starting the day after surgery.

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PATIENTS AND METHODS

Case Recognition

Clinicians participating in the study were instructed to be alert for the symptoms and signs associated with endophthalmitis such as pain; loss of vision; hypopyon; chemotic conjunctiva; and edema of the lids, conjunctiva, or cornea.

At least 1 follow-up visit was required for each patient between 3 and 6 weeks after surgery. Each surgeon used his or her clinical judgment at this visit and provided a positive or negative response to the diagnosis of presumed infective endophthalmitis. Patients seen within the first 2 weeks were tracked for 6 postoperative weeks.

Exhaustive data were collected on comorbidities (eg, diabetes). These have been reported in detail.[6]

The 29 patients who were diagnosed as having presumed infective endophthalmitis were immediately investigated by anterior chamber and vitreous taps for (1) Gram stain, (2) microbiology culture, and (3) polymerase chain reaction (PCR). A vitreous cutter was recommended for the vitreous tap. The sample size was not specified. Treatment of the endophthalmitis was at the discretion of the supervising surgeon. The Gram stains and microbiology cultures were performed locally using each center's hospital microbiology laboratory and a protocol (see below) produced for the study. The PCR tests were carried out centrally and replicated independently at 2 centers: the Institute of Molecular Biology, University of Regensburg, Germany, and Vissum-Instituto de Oftalmológico de Alicante, Alicante, Spain.

Any case in which 1 or more of these test procedures yielded a positive result was reclassified as proven infective endophthalmitis. If all test results were negative, the case was reclassified as nonproven infective endophthalmitis.

All nonproven cases were subsequently reviewed to establish whether any was associated with other patients in the unit with toxic anterior segment syndrome,[7] when 3 or more cases usually occurred together, or when there was phaco-toxic uveitis (often due to hypersensitivity to lens protein or a residue of ethylene oxide in the intraocular lens). Such noninfective cases often present acutely within 24 to 48 hours of surgery but normally respond well to treatment with corticosteroids.

A more difficult problem was posed by late-presenting cases of chronic granulomatous or saccular (bag) endophthalmitis, often due to infection with *Propionibacterium acnes*, coagulase-negative staphylococci, or diphtheroids. Participating clinicians were asked to report all these cases that presented within or after 6 weeks of surgery and for the cases to be included in the totals of presumed and/or proven infective endophthalmitis cases.

Investigation and Microbiology of Specimens

All laboratories taking part in this study were accredited or certified in their own country and took part in National Quality Control Schemes. Samples were submitted to the laboratory as masked samples without details of antibiotic prophylaxis.

Gram stain was performed on all samples and the result recorded. Semiquantitative culture of aqueous and vitreous samples was performed by streak cultures on 4% horse blood and chocolate agar plates and liquid enrichment

media such as brain–heart infusion (BHI) broth that support the growth of fungi. Plates were incubated aerobically at 37°C with 4% carbon dioxide (CO_2) for 4 days. Isolation of any bacterial colonies was deemed indicative of culture-positive endophthalmitis. Blood agar plates were also incubated anaerobically for 14 days, together with BHI broth, to culture *Propionibacterium*. If fungi were seen on Gram stain, Sabouraud agar plates would also be inoculated and incubated at 32°C for 14 days.

The bacterial colonies cultured on each agar plate were identified by the API strip test method, and an isolate was sent to a central laboratory (Roland J. Koerner, MD, MRCPath, Sunderland, United Kingdom) for antibiotic susceptibility testing. These were determined using the E-test with approved standards (Cambridge Diagnostic Services Ltd.). For testing *Staphylococcus* species, Iso-sensitest agar was used (Oxoid). Two to 4 colonies were picked from the purity plate and suspended in 5 mL water, which was poured onto the plates; the excess was removed to achieve a homogenous confluent growth as directed by the manufacturer. These plates were incubated in air at 35°C for 18 hours. For testing *Streptococcus* species, Iso-sensitest agar plus 5% defibrinated horse blood was used. Five to 10 colonies were picked from the purity plate and suspended in 5 mL water, which was poured onto the plates; the excess was removed to achieve a homogenous confluent growth. These plates were incubated at 35°C in air enriched with 5% CO_2 for 18 hours. All plates had been quality controlled to British Society for Antimicrobial Chemotherapy (BSAC) standards (Available at: http://www.bsac.org.uk. Accessed June 16, 2008).

Samples of aqueous and vitreous were stored for PCR for testing in a central laboratory at –20°C if there was to be any delay or at 4°C if courier transport was available the following day. Samples were initially sent to Udo Reischl, PhD (Regensburg, Germany).

Molecular Biology in Regensburg

Vitreous and/or aqueous humor from patients with suspected intraocular infection have been routinely tested with PCR in Regensburg since 1997. Bacterial broad-range 16S rDNA PCR has been established and carefully evaluated with 450 ocular specimens (mainly vitrectomy material and aqueous humor from adult eyes with acute and subacute inflammation) and more than 2000 other clinical specimens from normally sterile sites.[8]

At the time of surgery, the vitrectomy material was collected in sterile Eppendorf tubes. With respect to the high sensitivity of PCR-based procedures, special care was taken to avoid contamination of the specimen with extraneous bacterial or fungal DNA at the time of surgery, specimen collection, and throughout sample processing in the laboratory. The PCR analysis was conducted in 3 dedicated and physically separated rooms with HEPA air filtration for DNA extraction, PCR amplification, and PCR product analysis.

Total DNA was prepared from the vitreous and/or aqueous humor specimens according to previously published protocols[9,10] with minor modifications. Following the proteinase K digestion step of the QIAamp DNA minikit (Qiagen), 5 cycles of a freeze/thaw pretreatment were performed[10] to ensure efficient lysis of fungal or bacterial cell walls. The DNA preparation was eluted from the spin column with 50 µL of prewarmed (70°C) AE buffer (QIAamp DNA minikit) and divided into 4 aliquots with an extra fifth aliquot for the Alicante laboratory.

The first aliquot was subjected to 33 cycles of PCR using PCR-Master (Roche Diagnostics) and primer oligonucleotides DG 74, RW 01, and RDR080, specific for a conserved sequence within bacterial 16S rRNA genes. Primer sequences and amplification conditions are described in Greisen et al.[11] Different production lots of enzymatic and biochemical compounds were obtained from the manufacturers and pretested for traces of contaminating bacterial or fungal DNA. Lots with the lowest level of DNA contamination were used throughout the experiments. In the case of a positive PCR result but an inconclusive amplicon sequence, a second fragment of the bacterial 16S rRNA gene (V3 loop) was amplified with PCR primers BF (5′-ACTCCTACGGGAGG CAGCAGT-3′) and CR (5′-ACGTCATCCCCACCTTCCTC-3′) to obtain a further stretch of sequence information from the underlying bacterial organism.

The second aliquot of the DNA preparation was subjected to a PCR with primer oligonucleotides KWC348 (5′-ATG CATGTCTAAGTATAA-3′) and KWC 352 (5′-ACGGTAT CTGATCATCTT-3′), specific for the 18S small subunit ribosomal RNA gene of fungal organisms. The following thermal profile was applied in a Gene Amp PCR System 9700 (Applied Biosystems): initial denaturation at 95°C for 5 minutes, 35 cycles of 30 seconds at 94°C, 30 seconds at 63°C, and 60 seconds at 72°C, followed by a final 5-minute elongation step at 72°C.

The third aliquot was subjected to quantitative real-time PCR using the LightCycler Control Kit DNA (Roche Diagnostics). This kit provides all components for amplifying and specific detection of the human ß-globin gene, which is present in nearly every kind of human specimen. In the course of the present study, this assay was performed for monitoring of efficient sample preparation and the detection of Taq DNA polymerase inhibitors, possibly present in the DNA preparations from clinical samples.

The fourth aliquot of the DNA preparation was stored at −80°C for subsequent experiments or, when requested, subjected to PCR for *Toxoplasma gondii*[12] and/or viral pathogens according to previously published protocols.

At least 3 control samples were included in each series of PCR experiments. Negative control sample 1 (DNA extraction control) was prepared by replacing the clinical specimen with an equal volume of PCR grade water. Negative control sample 2 (amplification control) was prepared by replacing the DNA template with PCR grade water. The PCR positive control reaction was spiked with 10 ng of *Escherichia coli* DNA and processed as the last sample of each series.

The PCR reaction mixtures were electrophoretically separated in agarose gels and analyzed for the presence of specific amplification products. In the case of a positive PCR result, the nucleotide sequences of the amplified fragments were determined for a more precise differentiation of bacterial and fungal pathogens down to species level. After gel electrophoresis, amplification products of the expected size were extracted from the gel matrix using the HighPure PCR Product Purification kit (Roche Molecular Biochemicals) and subjected to direct sequencing of both strands with the dye-terminator strategy using an automated ABI Prism 310 DNA sequencer (Applied Biosystems). Sequencing reactions were performed with the PCR primer oligonucleotides used for amplification. Computer-assisted comparison of the nucleotide sequences determined from the PCR amplicons with all GenBank sequence entries deposited so far and/or the proprietary and quality-controlled SmartGene IDNS database (Available at: http://www.smartgene.com.

Accessed June 16, 2008) revealed a list of candidate species based on the level of sequence homologies.

Molecular Biology in Alicante

Extracted DNA (the fifth aliquot) and negative extract control were sent by courier from Regensburg to Alicante. It was amplified using a RoboCycler 96 Temperature Cycle (Stratagene). Primers used and PCR conditions are specified below.

Two control samples (plus negative extract control) were included in each series of PCR experiments. Negative control sample 1 (amplification control) was prepared by replacing the DNA template with PCR grade water. The PCR positive control reaction was spiked with 1 ng of *Staphylococcus epidermidis* DNA (for bacterial PCR) or *Candida albicans* DNA (for fungal PCR).

Two kinds of detection by PCR were performed, the first to amplify the bacteria 16S RNA gene and the second to amplify the ITS/5.8S RNA gene region of fungal DNA. The bacterial PCR was performed in 2 steps: The first PCR was performed as described by Hykin et al.[9] with some modifications. Briefly, the universal primers used for bacterial amplification were the primer 66f (5′-GGCGGCRKGCCTAA YACATGCAAGT-3)[9] and the primer 1147r (5′-GACAAC CATGCASCACCTGT-3′) (Life Technologies). The 25 μL PCR reaction mixture contained 5 μL DNA template: 2.5 μL PCR buffer (with magnesium chloride [$MgCl_2$]), 200 μM deoxynucleoside triphosphate, 25 pmol each primer, and 0.5 U of Taq DNA polymerase (Roche Diagnostics). Reactions were as follows: 1 cycle at 95°C for 5 minutes; 35 cycles with a denaturation step at 95°C for 30 seconds, annealing at 55°C for 1 minute, and final extension step at 72°C for 1 minute. The size of the amplified fragment was 954 bp and contained the hypervariable V3 region of the 16S rRNA gene.

For the second amplification, the primers used were, 1 of the primers of the first PCR, the primer 66f (5′-GGCGGCR KGCCTAAYACATGCAAGT-3),[9] and the primer 518r (5′-ATTACCGCGGCTGCTGG-3′)[13] (Life Technologies). Seminested reactions contained 1 μL first-round product in 25 μL PCR reaction mixture and contained 2.5 μL of PCR buffer (with $MgCl_2$), 200 μM deoxynucleoside triphosphate, 25 pmol of each primer, and 0.5 U of Taq DNA polymerase (Roche Diagnostics). Reactions were 1 cycle at 94°C for 4 minutes, 30 cycles with a denaturation-step at 94°C for 1 minute followed by annealing at 55°C for 1 minute and extension at 72°C for 2 minutes, and 1 final extension step at 72°C for 6 minutes.

The fungal PCR was performed as described by Ferrer et al.[14]

Ten-microliter aliquots of each amplified product were electrophoretically separated in a 2% agarose gel in 1 × Tris–borate–ethylenediaminetetraacetic acid buffer and visualized using ethidium bromide under ultraviolet illumination. Molecular weight ladders were included in each run (pBR322 DNA/BsuRI or Gene Ruler 100 bp DNA Ladder Plus, MBI Fermentas).

Amplified DNA from PCR was purified using the Geneclean II kit (BIO 101 Inc.) according to the manufacturer's instructions, and directly cycle sequenced in both directions using the BigDye terminators Ready Reaction Kit (PE Applied Biosystems) on an ABI prism automated DNA sequencer (model 377, version 2.1.1, Applied Biosystems Warrington). The primers used were 66f, 1147f, and 518f for bacterial DNA and ITS1 and ITS4 for fungal DNA. The sequence was analyzed using the BLAST alignment program of the GenBank database (National Institutes of Health) and Fasta3 program of the EMBL database.

RESULTS

Fourteen of 29 cases of presumed endophthalmitis were proven by culture as follows: 2 *Streptococcus pneumoniae*; 2 *Streptococcus salivarius*; 1 *Streptococcus oralis*; 1 *Streptococcus sanguis* (synonymous *Streptococcus sanguinis*); 7 *Staphylococcus epidermidis*; 1 *Staphylococcus warneri*.

In 6 of 29 cases of presumed endophthalmitis, the culture was negative but the PCR result was positive as follows (1 each): *Streptococcus suis*; *Streptococcus mitis* and *S epidermidis* (mixed infection); *Staphylococcus aureus* and *Propionibacterium acnes* (mixed infection); *S aureus*; *Staphylococcus hominis* or *haemolyticus*; *P acnes*.

Nine of 29 cases of presumed endophthalmitis did not yield a positive result by Gram stain, culture, or PCR. Possible reasons include late sampling, dry sampling, lost samples, or failure to take a sample.

Clinical data on all patients have been published.[6]

Table 1 compares the results for each sample for Gram stain, culture, and PCR (Regensburg and Alicante). There were no pathogenic fungi isolated or recognized by PCR in this study. However, with the PCR test, fungal contamination with nonpathogens occurred on 2 occasions and on 1 occasion with *Candida* species, thought to have arisen when the sample was prepared in Regensburg or transported and received in Alicante.

Tables 2 and 3 show the minimum inhibitory concentration (MIC) results for 11 of the 14 isolates. Three cultures were lost before the group could be tested together.

DISCUSSION

This study compared Gram stain, traditional culture, and molecular biology performed in 2 separate laboratories for diagnostic use on specimens of aqueous and vitreous from presumed cases of postoperative endophthalmitis. The Gram stain was least effective for aqueous specimens, being positive on only 1 of 11 occasions. Culture was positive on 7 of 11 occasions. The remaining 4 patients had a positive culture from the vitreous sample when the aqueous sample was negative. The Gram stain was more useful on vitreous specimens, being positive on 7 of 10 occasions. Culture was positive on 9 of 10 occasions. The remaining 1 patient had positive growth from the aqueous when the vitreous sample was negative. When possible, samples of aqueous and vitreous should be collected for the laboratory investigation of presumed postoperative endophthalmitis.

Table 1. Results in 29 patients with presumed postoperative endophthalmitis for each culture and PCR (Regensburg and Alicante).

Description	Specimen	Gram Stain for Bacteria	Culture	Molecular Biology Regensburg	Molecular Biology Alicante
Cases of endophthalmitis with positive culture					
Pt 10	Aqueous	Negative	*Streptococcus pneumoniae*	Not tested	Not tested
	Vitreous	Positive: cocci	*Streptococcus pneumoniae*	Not tested	Not tested
Pt 22	Aqueous	Negative	*Streptococcus pneumoniae*	Sample lost by courier en route to Regensburg	Sample lost by courier en route to Regensburg
	Vitreous	Positive: cocci	*Streptococcus pneumoniae*	Sample lost by courier en route to Regensburg	Sample lost by courier en route to Regensburg
Pt 3	Aqueous	Negative	No growth	Not tested	Not tested
	Vitreous	Positive: cocci	*Streptococcus oralis*	Positive: *Streptococcus mitis/S sanguini / S oralis/S mitis* group	Positive: *Streptococcus mitis/S sanguinis/ S oralis / S mitis* group
Pt 21	Aqueous	Not tested	*Streptococcus salivarius*	Not tested	Not tested
	Vitreous	Positive: cocci	*Streptococcus salivarius*	Not tested	Not tested
Pt 27	Aqueous	Negative	*Streptococcus sanguis*	Positive: *Streptococcus suis* group (refer to text)	Negative
	Vitreous	Positive: cocci	Mixed α-hemolytic streptococci	Positive: *Streptococcus suis* group (refer to text)	Positive: *Streptococcus* spp
Pt 28	Aqueous	Negative	*Streptococcus salivarius*	Positive: *Streptococcus salivarius* or *Streptococcus vestibularis*	Positive: *Streptococcus salivarius* or *Streptococcus mitis*
	Vitreous	Negative	No growth	Weak positive: *Streptococcus salivarius* or *Streptococcus vestibularis*	Negative
Pt 4	Aqueous	Negative	No growth	Positive for eubacterial primers, but sequence reactions not interpretable	Positive for eubacterial primers, but sequence reactions not interpretable
	Vitreous	Positive: cocci	*Staphylococcus warneri*	Positive for eubacterial primers, but sequence reactions not interpretable	Positive for eubacterial primers, but sequence reactions not interpretable
Pt 8	Aqueous	Negative (pus cells +++)	*Staphylococcus epidermidis*	Negative: β-globin gene positive, indicating human DNA acting as an inhibitor	Negative by 2 methods
Pt 14	Aqueous	Positive: bacteria (cocci)	*Staphylococcus epidermidis*	Positive: *Staphylococcus epidermidis*	Positive: *Staphylococcus epidermidis*
	Vitreous	Positive: bacteria (cocci)	*Staphylococcus epidermidis*	Positive: *Staphylococcus epidermidis*	Positive: *Staphylococcus epidermidis*
Pt 20	Aqueous	Negative	*Staphylococcus epidermidis*	Not tested	Not tested
Pt 25	Aqueous	Not tested	No growth	Positive: *Staphylococcus epidermidis*	Positive: *Staphylococcus epidermidis*
	Vitreous	Not tested	*Staphylococcus epidermidis*	Positive: *Staphylococcus epidermidis*	Positive: *Staphylococcus epidermidis*
Pt 17	Vitreous	Negative	*Staphylococcus epidermidis*	Not tested	Not tested
Pt 12	Aqueous	Negative	No growth	Not tested	Not tested
	Vitreous	Negative	*Staphylococcus epidermidis*	Not tested	Not tested

(*continued on next page*)

Table 1 (Cont.)

Description	Specimen	Gram Stain for Bacteria	Culture	Molecular Biology Regensburg	Molecular Biology Alicante
Pt 9	Aqueous	Negative	No growth	Negative	Weak positive for eubacterial primers but insufficient to sequence
	Vitreous	Not tested	*Staphylococcus epidermidis* (methicillin resistant)	Positive: *Staphylococcus epidermidis*	Positive - *Staphylococcus epidermidis*
Cases of endophthalmitis with negative culture but a positive PCR					
Pt 2	Aqueous	Negative	No growth	Positive: *Streptococcus suis, S. salivarius* or *Streptococcus spp.*	Positive for eubacterial primers but insufficient to sequence
Pt 6	Aqueous	Negative	No growth	Positive: *Staphylococcus epidermidis, Gemella haemolysans*	Positive: *Staphylococcus epidermidis*
	Vitreous	Negative	No growth	Positive: *Streptococcus mitis, Staphylococcus epidermidis*	Positive: *Staphylococcus epidermidis*
Pt 5	Aqueous	Negative	No growth	Positive: *Staphylococcus aureus, Propionibacterium acnes**	Weak positive for eubacterial primers, but insufficient to sequence
	Vitreous	Negative	No growth	Positive: *Staphylococcus aureus, Staphylococcus epidermidis, P acnes**	Weak positive for eubacterial primers. but insufficient to sequence
Pt 24	Aqueous	Negative	No growth	Negative: repeated with Light Cycler Staphylococcus PCR test kit—positive for *S aureus* or coagulase-negative staphylococci	Positive: *Staphylococcus aureus*
Pt 18	Vitreous	Negative	No growth	Positive: *Staphylococcus hominis* or *Staphylococcus haemolyticus*	Positive: *Staphylococcus hominis* or *Staphylococcus lugdunensis* or *Staphylococcus hemolyticus*
Pt 29	Aqueous	Negative	No growth	Positive: *Propionibacterium acnes*	Positive: unable to sequence
	Vitreous	Negative	No growth	Weak positive: *Propionibacterium* spp; specific PCR assay: weak positive	Negative
Cases of nonproven presumed endophthalmitis					
Pt 26	Aqueous	Negative	No growth	Negative	Negative
	Vitreous	Negative	No growth	Negative; repeated with Light Cycler *Staphylococcus* PCR test kit with negative result	Positive for *Staphylococcus lugdenensis* (but repeat test negative and repeat sample from Regensburg negative)

(*continued on next page*)

Table 1 (Cont.)

Description	Specimen	Gram Stain for Bacteria	Culture	Molecular Biology Regensburg	Molecular Biology Alicante
Pt 23	Aqueous	Negative	No growth[†]	Negative	Negative
	Vitreous	Negative	No growth[†]	Negative	Negative
Pt 19	Aqueous	Negative	No growth	Not tested	Not tested
	Vitreous	Negative	No growth	Negative	Negative
Pt 16	Aqueous	Not tested	No growth	Negative	Negative
Pt 15	Aqueous	Negative	No growth	Negative	Negative
	Vitreous	Negative	No growth	Negative	Negative
Pt 13	Aqueous	Negative	No growth	Negative	Negative
	Vitreous	Negative	No growth	Negative	Negative
Pt 11	Aqueous	Negative	No growth	Not tested	Not tested
	Vitreous	Negative	No growth	Negative	Negative
Pt 7	Aqueous	Negative	No growth	Negative	Negative
	Vitreous	Negative	No growth	Negative	Negative
Pt 1	Aqueous	Negative	No growth	Negative	Negative
	Vitreous	Negative	No growth	Not tested	Not tested
Additional cases with presumed endophthalmitis[‡]					
Pt A	Vitreous	Negative	*Streptococcus pneumoniae*	Positive: *Streptococcus pneumoniae* (*S pneumoniae* Light Cycler PCR assay positive; *S pneumoniae* lytA-Gen test positive)	Positive - *Streptococcus pneumoniae*
Pt B	Aqueous	Negative	No growth	Positive: - *Proteus mirabilis*	Weak positive: insufficient DNA to sequence
	Vitreous tap	Positive: gram-negative rod	No growth	Positive: - *Proteus mirabilis*	Positive: *Proteus mirabilis*
	Vitreous fluid	Positive: gram-negative rod	*Proteus mirabilis*	Positive: *Proteus mirabilis*	Positive: *Proteus mirabilis*
Pt C	Aqueous	Negative	No growth	Not tested	Not tested
	Vitreous	Negative	No growth	Negative	Negative

PCR = polymerase chain reaction; Pt = patient; spp = species
*Specimen tubes broke in transport to Regensburg.
[†]Samples were collected 2 weeks after the eye was treated with antibiotics.
[‡]Patients A (endophthalmitis), B (endophthalmitis), and C (postoperative uveitis, noninfective) were not part of the European study but were evaluated with similar methods and are included for comparison.

The 2 molecular biology laboratories used different methods to identify bacterial and fungal DNA. Their overall results, however, closely tallied with each other, and there would appear to be little advantage in doubling the cost and effort by processing specimens in 2 molecular biology laboratories for routine analysis rather than using 1 laboratory.

Seven samples of aqueous from the 14 culture-positive patients were analyzed by PCR, and 5 of 7 were positive. In 2 cases, however, 1 molecular biology laboratory was negative when the other was positive and vice versa. Overall, PCR was much better than the Gram stain (1 of 11 positive) for identifying the bacterial pathogen in aqueous specimens. Five vitreous specimens from the 14 culture-positive patients were tested by PCR. All 5 were positive in 1 molecular biology laboratory and 4 of 5 positives were positive in the other laboratory. The difference was a weak positive result in 1 laboratory interpreted as negative in the other laboratory; interestingly, the bacterium was isolated from the aqueous but not from the vitreous sample. If a bacterium has been isolated from the aqueous or vitreous, there is little advantage in also performing PCR.

Polymerase chain reaction confirmed the laboratory diagnosis in 6 cases of presumed endophthalmitis that were Gram stain and culture negative. Both molecular biology laboratories found the samples positive for bacteria for aqueous and/or vitreous samples.

Table 2. Nonquinolone antibiotic MIC results for 11 of 14 isolates.

Isolate	Penicillin	Oxacillin	Ampicillin	Cefuroxime
S pneumoniae	0.016 (S)	0.064 (S)	<0.016 (S)	0.032 (S)
S pneumoniae	0.032 (S)	0.064 (S)	<0.016 (S)	0.032 (S)
S salivarius	0.38 (R)	1.0 (R)	0.125 (S)	0.098 (S)
S salivarius	0.25 (R)	1.5 (R)	0.125 (S)	0.125 (S)
S oralis	0.094 (S)	0.5 (R)	0.016 (S)	1.0 (I)
S sanguis	2.0 (R)	8.0 (R)	0.75 (R)	1.5 (R)
St epidermidis	4.0 (R)	0.125 (S)	0.5 (S)	0.5 (S)
St epidermidis	6.0 (R)	1.0 (R)	1.5 (S)	3.0 (R)
St epidermidis	0.38 (R)	0.125 (S)	0.094 (S)	0.38 (S)
St epidermidis	12 (R)	0.75 (R)	1.5 (S)	1.5 (I)
St epidermidis	8.0 (R)	1.0 (R)	2.0 (I)	3.0 (R)
Breakpoint values				
BSAC				
*St aureus**	≤0.06	≤0.06	≤0.06	≤4.0†
Staphylococcus spp	0.12	0.12	2.0	≤4.0†
S pneumoniae	≤0.06	≤0.06	≤0.06	≤0.5
α-hemolytic *Streptococcus* spp	≤0.12	NA	≤1.0	NA
CLSI				
*St aureus**	—	≤4.0	≤0.25	≤8.0
Staphylococcus spp	0.12	≤0.25	≤0.25	≤8.0
S pneumoniae	0.06	≤0.12	≤0.12	≤0.12
α -hemolytic *Streptococcus* spp	0.12	—	≤0.25	≤1.0
DIN				
*St aureus**	≤0.125	≤1.0	≤0.125	≤4.0
Staphylococcus spp	≤0.125	≤1.0	≤0.125	≤4.0
S pneumoniae	≤0.125	≤1.0	≤2.0	≤4.0
α-hemolytic *Streptococcus* spp	≤0.125	≤1.0	≤2.0	≤4.0

BSAC = British Society for Antimicrobial Chemotherapy; CLSI = Clinical and Laboratory Standards Institute; DIN = Deutsches Institut für Normung; (I) = intermediate; NA = not available; (R) = resistant; (S) = sensitive; spp = species

*_St aureus_: MSSA, β-lactamase negative

†Value is estimated.

Although there was similar specific identification of the pathogen by sequencing, minor differences occurred. These were thought to be due to inhibitors within the sample, such as human DNA from pus cells, or to the fact that 1 laboratory (Regensburg) received the specimen, sending an aliquot of extracted DNA to the second laboratory (Alicante).

One disadvantage of molecular diagnosis is the lack of standardization between laboratories because these recently developed techniques are not optimized. This was a blind study performed at 2 different laboratories; both used PCR for the microbiological diagnosis, although they used different equipment and amplification conditions. The results show 86% correlation for microbial DNA detection, 85% in aqueous samples and 87% in vitreous samples. The other 14% corresponds to samples that were positive in 1 laboratory and negative in the other laboratory. This variation is because we were reaching the minimum detection levels of the methods. The size of sample that is usually obtained for intraocular infection diagnosis is very small (microliters); moreover, in this study, it was divided in various aliquots for different methods of diagnosis (stain, culture, and 2 molecular laboratories). Furthermore, the conditions of PCR used for each laboratory (primers, cycles, and temperature) were different and it is possible that some conditions were more sensitive than others for DNA amplification.[15,16] However, the results obtained show that PCR is a powerful tool to detect intraocular infection.

Specimens from 9 patients with presumed endophthalmitis were negative by Gram stain, culture, and PCR in both laboratories. This included 9 samples of aqueous and 8 of vitreous. Possible reasons vary from late collection of the sample (patient 23, Table 1), a dry vitreous tap, attempted vitreous extraction with a syringe and needle instead of a guillotine cutter, and that the case was genuinely inflammatory (postoperative uveitis, patient C, Table 1) and not infected. Collection of the sample after an intravitreal injection

Table 2. (Cont.)

Tetracycline	Chloramphenicol	Rifampicin	Azithromycin	Gentamicin	Vancomycin
0.094 (S)	1.5 (S)	0.01 (S)	64.0 (R)	6 (R)	0.5 (S)
0.125 (S)	2.0 (S)	0.016 (S)	1.5 (I)	12 (R)	0.75 (S)
0.19 (S)	2.0 (S)	0.016 (S)	32.0 (R)	3 (R)	0.75 (S)
0.38 (S)	2.0 (S)	0.064 (S)	0.75 (S)	4 (R)	0.75 (S)
0.38 (S)	2.0 (S)	0.094 (S)	0.75 (S)	16 (R)	0.75 (S)
0.125 (S)	1.0 (S)	0.047 (S)	0.38 (S)	3 (R)	0.5 (S)
0.5 (S)	1.5 (S)	<0.016 (S)	6.0 (R)	0.5 (S)	1.5 (S)
0.125 (S)	1.5 (S)	<0.016 (S)	>256.0 (R)	0.5 (S)	1.5 (S)
0.094 (S)	1.5 (S)	<0.016 (S)	6.0 (R)	0.5 (S)	1.5 (S)
0.125 (S)	1.5 (S)	<0.016 (S)	6.0 (R)	0.5 (S)	1.5 (S)
0.19 (S)	3.0 (S)	<0.016 (S)	8.0 (R)	0.5 (S)	2.0 (S)
≤1.0	≤8.0	≤1.0	≤1.0	—	≤4.0
≤11.0	≤8.0	8.0	≤1.0	≤1.0	≤4.0
≤1.0	≤8.0	≤1.0	≤1.0	≤1.0	≤4.0
≤1.0	NA	NA	NA	NA	NA
≤4.0	≤8.0	≤1.0	≤2.0	≤4.0	≤2.0
≤4.0	≤8.0	≤1.0	≤2.0	≤4.0	≤2.0
≤2.0	≤4.0	≤1.0	≤0.5	NA	≤1.0
≤2.0	≤4.0	NA	≤0.5	NA	≤1.0
≤1.0	≤8.0	NA	≤2.0	≤1.0	≤4.0
≤1.0	≤8.0	NA	≤2.0	≤1.0	≤4.0
≤1.0	≤8.0	NA	≤2.0	≤1.0	≤4.0
≤1.0	≤8.0	AN	≤2.0	≤1.0	≤4.0

of antibiotic agent can be expected to yield a negative culture result. The presence of polymorphonuclear (pus) cells can yield human DNA that acts as an inhibitor in the PCR test (patient 8, Table 1). In such circumstances, the Gram stain may identify dead bacteria, often within polymorphonuclear cells; however, it was negative in the 8 samples tested (Table 1).

Three further patients with presumed postoperative endophthalmitis, but not included in the ESCRS study, were included for comparison with similar methods (Table 1). The first case yielded a negative Gram stain from the vitreous when *S pneumoniae* was cultured; PCR was also positive for *S pneumoniae* in both molecular biology laboratories. The second case had a negative Gram stain from the aqueous, but 2 samples of vitreous were positive with gram-negative rods. *Proteus mirabilis* was cultured from only 1 of 3 samples (vitreous fluid) but was confirmed by PCR in all 3 samples by both molecular biology laboratories. The third case was negative by Gram stain, culture, and PCR in both laboratories and was later thought to be due to noninfective postoperative uveitis. Including these 3 nonstudy patients provides additional information.

Overall, both an aqueous tap and vitreous sample should be collected from patients with presumed postoperative endophthalmitis. Separate aliquots of both aqueous and vitreous should be stored in Eppendorf tubes at −20°C for later PCR. If Gram stain and culture (taking 10 minutes and 1 night, respectively) are positive, there is little advantage in performing PCR as well. However, if both samples are negative, PCR with broad-range primers becomes very useful, particularly as it not only confirms bacterial or fungal infection, but can also be expected to identify the pathogenic species by sequencing.

In addition to the isolation and identification of the pathogen(s) causing endophthalmitis, the diagnostic microbiology laboratory will be expected to determine the antimicrobial susceptibilities and provide guidance on the choice of antibiotic agent based on the

Table 3. Quinolone antibiotic MIC results for 11 of 14 isolates.

Isolate	Ciprofloxacin	Levofloxacin	Gatifloxacin	Moxifloxacin
S pneumoniae	0.5 (R)	0.5 (S)	0.25 (S)	0.094 (S)
S pneunmoniae	0.75 (R)	0.75 (S)	0.25 (S)	0.094 (S)
S salivarius	0.5 (R)	0.75 (S)	0.19 (S)	0.094 (S)
S salivarius	0.75 (R)	1.0 (S)	0.25 (S)	0.125 (S)
S oralis	6.0 (R)	1.5 (S)	0.5 (S)	0.25 (S)
S sanguis	0.75 (R)	0.75 (S)	0.38 (S)	0.094 (S)
St epidermidis	0.125 (S)	0.125 (S)	0.094 (S)	0.047 (S)
St epidermidis	6.0 (R)	6.0 (R)	2.0 (R)	0.75 (S)
St epidermidis	0.125 (S)	0.125 (S)	0.125 (S)	0.064 (S)
St epidermidis	4.0 (R)	3.0 (R)	1.0 (I)	0.75 (S)
St epidermidis	4.0 (R)	6.0 (R)	1.5 (R)	0.5 (S)
Overall sensitivity	18% (2/11)	73% (8/11)	73% (8/11)	100% (11/11)
Breakpoint values				
BSAC				
*St aureus**	≤1.0	NA	≤1.0	≤0.5
Staphylococcus spp	≤1.0	NA	≤1.0	NA
S pneumoniae	≤0.12	≤2.0	≤1.0	≤0.5
α-hemolytic *Streptococcus* spp	NA	NA	NA	NA
CLSI				
*St aureus**	≤1.0	≤1.0	≤1.0	≤0.5
Staphylococcus spp	≤1.0	≤1.0	≤0.5	≤0.5
S pneumoniae	NA	≤2.0	≤1.0	NA
α-hemolytic *Streptococcus* spp	NA	≤2.0	≤1.0	NA
DIN				
*St aureus**	≤1.0	≤2.0	≤1.0	≤1.0
Staphylococcus spp	≤1.0	≤2.0	≤1.0	≤1.0
S pneumoniae	≤1.0	≤2.0	≤1.0	≤1.0
α-hemolytic *Streptococcus* spp	≤1.0	≤2.0	≤1.0	≤1.0

BSAC = British Society for Antimicrobial Chemotherapy; CLSI = Clinical and Laboratory Standards Institute; DIN = Deutsches Institut für Normung; (I) = intermediate; NA = not available; (R) = resistant; (S) = sensitive; spp = species
**St aureus*: MSSA, β-lactamase negative

results. Close liaison between the ophthalmologist and local microbiology department will enhance optimal management.

The MICs for bacteria isolated in the ESCRS study are shown in Tables 2 and 3 using 3 schemes as defined by BSAC (Available at: http://www.bsac.org.uk. Accessed June 16, 2008), Clinical and Laboratory Standards Institute,[17] or the Deutsches Institut für Normung.[18] All isolates of streptococci and staphylococci were sensitive to vancomycin, which, together with ceftazidime for effectiveness against gram-negative rods, remains the antibiotic agent of choice for intravitreal therapy of endophthalmitis.[15,16] As expected, all isolates of streptococci were resistant to gentamicin.[15,16] Two isolates of streptococci and 2 of *S epidermidis* were resistant to cefuroxime but there were no cases of endophthalmitis associated with methicillin-resistant *S aureus* (MRSA). All isolates remained sensitive to tetracycline, rifampicin, and chloramphenicol. Three of 6 isolates of streptococci and all 5 isolates of *S epidermidis* were resistant to azithromycin. This is similar to a large study of community-based eye infections (conjunctivitis and keratitis) when *S epidermidis* was usually found to be resistant to erythromycin,[19] limiting the potential usefulness of this antibiotic agent, which is, however, effective against *P acnes*. There were no isolations of gram-negative bacteria or fungi.

The overall sensitivity of the 11 isolates to ciprofloxacin, levofloxacin, gatifloxacin, and moxifloxacin was 18%, 73%, 73%, and 100%, respectively. Levofloxacin had similar MIC values to those of gatifloxacin; moxifloxacin had the lowest MIC values. The use of levofloxacin for prophylaxis, reserving moxifloxacin for therapy of resistant keratitis, has been justified on the basis that all 6 isolates of streptococci were sensitive to it; similar results were found by Kresken and Behrens-Baumann.[19] However, 3 of 5 isolates of *S epidermidis* were resistant to levofloxacin; thus, future trends will have to be observed. Oral or intravitreal moxifloxacin are not used in Europe. There were no isolations of *S aureus*, although 2 cases of

endophthalmitis were identified as being due to *S aureus* by PCR; there were no cases of MRSA.

Antimicrobial therapy can be optimized by applying the relevant pharmacokinetic (PK)/pharmacodynamic (PD) parameters of the antibiotic(s) chosen.[15,16] This will be achieved by giving antibiotic agents, which are known to be nonirritating when injected intracamerally or intravitreally[15,16] because the blood–ocular barriers will not permit ready diffusion into the eye. This has been reviewed in the ESCRS Guidelines on Endophthalmitis (Available at: http://www.escrs.org/publications/eurotimes/07oct/eumatters.pdf. Accessed June 16, 2008). The relevant PK/PD parameter for suitable β-lactam antibiotic agents such as cefuroxime, ceftazidime, and vancomycin, a glycopeptide antibiotic, is "Time > MIC"; that is, the cumulative percentage of time over a 24-hour period when the antibiotic agent exceeds the MIC under steady-state kinetic conditions. For cefuroxime, this can be further enhanced by additional intravenous administration due to the high ratio of serum/aqueous humor/vitreous concentrations in the inflamed eye.[16] The bactericidal action of the fluoroquinolone antibiotics is related to the peak concentration C_{max} and the area-under-concentration time curve (AUC) over 24 hours divided by the MIC (AUC/MIC [h]).[20] Topical and oral systemic administration achieve concentrations sufficiently high to meet the PK/PD criteria.[15,16,21] Therefore, systemic and topical administration of a fluoroquinolone might be the only further antibiotic therapy required versus repeated intravitreous injections depending on the sensitivity (MIC) of the bacterium.

The intravitreal administration of aminoglycosides results in a high peak concentration meeting a high C_{max}/MIC ratio, the governing PK/PD parameter for the aminoglycosides. As exposure of bacteria to a high C_{max} is characterized by a prolonged postantibiotic effect, usually only 1 dose should be required. This therapeutic approach would not necessarily be supported by using susceptibility results obtained by simple disk-diffusion methods defined by BSAC (Available at: http://www.bsac.org.uk. Accessed June 16, 2008), Clinical Laboratory Standards Institute (Available at: http://www.nccls.org. Accessed June 16, 2008), or Deutsches Institut für Normung (Available at: http://www.din.de. Accessed June 16, 2008). As can be seen in Tables 2 and 3, the recommended values vary or no recommendation is given. For instance, the BSAC does not provide breakpoints for α-hemolytic streptococci. In these cases, or when an uncommon pathogen such as *Sphingomonas paucimobilis* is isolated,[22] the application of susceptibility results obtained by disk-diffusion methods may lead to therapeutic failure for reasons explained above.

In conclusion, this study has shown that the combination of culture-based and advanced molecular biology diagnostic methods optimizes identification of the etiological infective agent. We consider this study is unique in comparing cultures and PCR techniques; therefore, diagnostic microbiology laboratories should establish collaboration with centers providing molecular biology diagnostic methods. Determining the MIC rather than relying on susceptibility results obtained by the disk-diffusion method will enable the clinical microbiologist to optimize acute and postoperative antibiotic therapy.

APPENDIX

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