GUSTAVO BARRETO DE MELO

ENDOFTALMITE BACTERIANA: ASPECTOS EPIDEMIOLÓGICOS E DIAGNÓSTICOS

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina para obtenção do Título de Doutor em Ciências

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DEDICATÓRIA

A meus pais, irmãos e toda a família

A minha esposa

A todos que se dedicam ao progresso da ciência

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SUMÁRIO

LISTA DE ABREVIATURAS E SÍMBOLOS

%	Porcentagem ou percentagem
°C	Graus Celsius
ATL	Tampão tecidual de lise, do inglês "Tissue lysis buffer"
AUC	Área sob a curva, do inglês "Area Under the Curve"
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CoNS	"Coagulase-negative *Staphylococcus*"
CF	Conta dedos, do inglês "Counting finger"
CSLI	"Clinical and Standards Laboratory Institute"
Ct	Limiar de ciclos, do inglês "Cycle threshold"
dATP	Trifosfato de deoxiadenosina, do inglês "Deoxyadenosine triphosphate"
dCTP	Trifosfato de deoxicitidina, do inglês "Deoxycytidine triphosphate"
dGTP	Trifosfato de deoxiguanosina, do inglês "Deoxyguanosine triphosphate"
DNA	Ácido deoxirribonucléico, do inglês "Deoxyribonucleic acid"
dUTP	Trifosfato de deoxiuridina, do inglês "Deoxyuridine triphosphate"
FAPESP	Fundação de Amparo à Pesquisa de São Paulo
GN	Gram-negativo
GP	Gram-positivo
HCl	Cloreto de hidrogênio
HM	Movimento de mãos, do inglês "Hand motion"
IOL	Lente intraocular, do inglês "Intraocular lens"
IRB	Comitê de Ética em Pesquisa, do inglês "Institutional Review Board"
KCl	Cloreto de potássio
LEMC	Laboratório Especial de Microbiologia Clínica
LOFT	Laboratório de Oftalmologia
LP	Percepção luminosa, do inglês "Light perception"
mg	Miligrama
MgCl	Cloreto de magnésio
MGST-PCR	Análise em cadeia de polimerase múltipla baseada no sistema TaqMan, específica para Gram, do inglês "Multiplex Gram-Specific TaqMan-Based PCR"

MIC	Concentração inibitória mínima, do inglês "Minimum inhibitory concentration"
µl	Microlitro
µM	Micromolar
n	Número amostral
NA	Não-aplicável, do inglês "Not applicable"
NLP	Sem percepção luminosa, do inglês "No light perception"
NS	Não-suscetível, do inglês "Not susceptible"
PCR	Reação em cadeia de polimerase, do inglês "Polymerase chain reaction"
PCR-RFLP	Análise em cadeia de polimerase – Polimorfismo do Tamanho do Fragmento de Restrição, do inglês "Restriction fragment length polymorphism-PCR"
PPV	Vitrectomia via pars plana, do inglês "Pars plana vitrectomy"
RNA	Ácido ribonucléico, do inglês "Ribonucleic acid"
S	Suscetível, do inglês "Susceptible"
SCoN	*Staphylococcus* coagulase-negativa
SD	Desvio-padrão, do inglês "Standard deviation"
SGRU-PCR	Análise em reação de cadeia de polimerase universal baseada no SYBR Green 16S rDNA, do inglês "SYBR Green 16S rDNA-Based Universal PCR"
SPSS	Statistical Package for the Social Sciences
UNIFESP	Universidade Federal de São Paulo

RESUMO

Objetivos: I- Relatar a incidência de endoftalmite bacteriana e a frequência dos micro-organismos identificados numa instituição brasileira. **II-** Avaliar a frequência de micro-organismos isolados de pacientes com endoftalmite bacteriana e sua suscetibilidade antimicrobiana. **III-** Determinar a aplicabilidade da reação em cadeia de polimerase em tempo real (PCR em tempo real) no diagnóstico de endoftalmite bacteriana pós-operatória em casos diagnosticados clinicamente, e avaliar a presença de DNA bacteriano em amostras-controles.
Métodos: I- Todos os casos com diagnóstico clínico de endoftalmite pós-operatória de 2002 a 2008 foram incluídos nesta análise retrospectiva. Foram avaliados: número de cirurgias de catarata, incidência de endoftalmite, positividade dos exames laboratoriais de bacterioscopia e cultura de aquoso e vítreo e os resultados dos antibiogramas. **II-** Foi feita análise retrospectiva de prontuários de pacientes com suspeita diagnóstica de endoftalmite bacteriana. As seguintes informações foram avaliadas: número de casos com diagnóstico clínico de endoftalmite e com cultura positiva, fatores predisponentes para a infecção, bacterioscopia e cultura (aquoso e/ou vítreo), caracterização microbiológica e sua frequência, bem como suscetibilidade antimicrobiana. **III-** Foram incluídas amostras de olhos de pacientes com diagnóstico clínico de endoftalmite, de provável origem infecciosa, após cirurgia de catarata, assim como amostras de vítreo e de aquoso de olhos sem infecção ou reação inflamatória para controle. Foi realizada PCR em tempo real universal e específica para Gram bem como foram determinados sensibilidade e limiar de ciclos (Ct) da reação. Também foram realizadas bacterioscopia e cultura para as amostras clínicas.
Resultados: I- Setenta e três olhos de 73 pacientes (43 do sexo feminino e 30 do masculino) desenvolveram inflamação ocular de provável etiologia infecciosa após 24.590 cirurgias de catarata. A incidência reduziu-se de 0,49% em 2003 para 0,17% em 2006 e estabilizou-se nos anos subsequentes. *Staphylococcus* coagulase-negativa (SCoN) e *Streptococcus viridans* (56,5% e 15%, respectivamente) foram as bactérias mais comuns. Cultura e bacterioscopia foram negativas em 36,9%. SCoN apresentou taxas de sensibilidade de 80% à oxacilina, 90% às quinolonas de quarta geração e 100% à vancomicina. **II-** Cento e sete (46%) de 231 pacientes com endoftalmite bacteriana tiveram resultados positivos por bacterioscopia ou cultura. Desses, 97 (42%) pacientes tiveram positividade apenas na cultura. A maioria (62%) foi decorrente de procedimento cirúrgico (pós-operatório), 12% após trauma e 26% de fonte desconhecida. Foram isolados 100 micro-organismos (38 amostras de aquoso e 67 de vítreo) de 97 casos positivos na cultura. SCoN foram os mais frequentemente isolados (48%), seguidos por *Streptococcus viridans* (18%) e *Staphylococcus aureus* (13%). A suscetibilidade antimicrobiana para SCoN foi: vancomicina - 100%, cefalotina – 97,9%, cloranfenicol – 91,8%, amicacina – 91,6%, moxifloxacino – 89,5%, tobramicina – 85,4% gatifloxacino – 79,5%, gentamicina – 72,9%, ofloxacino – 70,8%, ciprofloxacino – 62,5%, oxacilina – 58,3%, ceftriaxona – 50% e penicilina – 33,3%. **III-** Onze pacientes com endoftalmite infecciosa (9 amostras de vítreo e 7 de aquoso) após cirurgia de catarata foram incluídos, assim como 12 amostras de vítreo e 50 de aquoso de olhos-controles. Foi possível identificar 80% e 70% dos pacientes com endoftalmite infecciosa por meio de bacterioscopia e cultura, respectivamente. PCR em tempo real foi positiva em 91% dos pacientes utilizando-se amostras de aquoso e/ou vítreo. Nenhum dos 12 vítreos-controles foi positivo por PCR em tempo real. Duas das amostras-controles de aquoso foram positivas. O ponto de corte do limiar de ciclos para PCR universal foi 36 (sensibilidade: 93,8%; especificidade: 100%) e 38 para PCR gram-específico (sensibilidade: 93,8%; especificidade: 100%). Micro-organismos gram-positivos predominaram e a acuidade visual variou de acordo com a bactéria causadora.
Conclusões: I- A incidência de endoftalmite bacteriana, os micro-organismos isolados e a

sensibilidade aos antibióticos estão em acordo com a literatura. Apesar do uso profilático de colírio antibiótico, casos de infecção foram identificados em bactérias sensíveis aos antibióticos usados topicamente. **II-** Bactérias gram-positivas foram as principais causas de endoftalmite infecciosa. SCoN foi o isolado mais comum e a suscetibilidade à oxacilina e às quinolonas de quarta geração foi menor do que relatado na literatura. **III-** PCR em tempo real é um método diagnóstico rápido e sensível nos casos de endoftalmite bacteriana. Sendo um método quantitativo, também pode servir para uma nova e distinta aplicação: diferenciação entre contaminação e infecção com base nos valores de limiar de ciclos.

Descritores: endoftalmite; epidemiologia; diagnóstico; reação em cadeia de polimerase; testes de sensibilidade microbiana.

INTRODUÇÃO

Endoftalmite é definida como uma inflamação intraocular. No entanto, rotineiramente, esse termo refere-se à inflamação decorrente de infecção intraocular. Apesar de rara, é uma condição potencialmente destrutiva para o olho, resultando em um grande prejuízo visual para a maioria dos pacientes e até mesmo atrofia do globo ocular[1].

Pode ser classificada em quatro formas, de acordo com sua origem: 1) pós-operatória (surgimento agudo e tardio, associada a cirurgias de catarata, glaucoma, transplante de córnea, entre outras), 2) endógena, 3) pós-traumática e 4) miscelânea (secundária a ceratite microbiana, por exemplo)[2]. Outra classificação mais ampla a considera apenas como endógena ou exógena. Esta ocorre após cirurgia intraocular, trauma penetrante, úlcera corneana, ou através de quebra de alguma barreira periocular pela qual penetram micro-organismos infectantes. Frequentemente, a forma exógena é subsequente à cirurgia de catarata e, na grande maioria dos casos, é causada por micro-organismos provenientes da microbiota ocular exógena[3]. Essas infecções são incomuns, com uma incidência que varia de 0,05 a 0,32%[1,4,5]. Já a endoftalmite endógena ocorre quando o micro-organismo chega ao olho pela corrente sangüínea, atravessando a barreira hemato-ocular. É menos comum que a exógena e ocorre em 2 a 6% dos casos de endoftalmite[1,6].

A endoftalmite exógena aguda, na maioria das vezes, está relacionada a procedimentos cirúrgicos e ocorre até seis semanas após sua realização[7]. Os principais micro-organismos envolvidos após a cirurgia da catarata (facectomia) são os *Staphylococcus* coagulase-negativa, principalmente *Staphylococcus epidermidis*. Bactérias gram-negativas e anaeróbias correspondem a uma parcela menor. A fonte mais provável da infecção é a microbiota conjuntival e palpebral do próprio paciente, que penetra o olho no momento da cirurgia[7]. Um estudo mostrou que a incidência de cultura positiva do humor aquoso pode atingir 29% no momento da cirurgia. No entanto, a incidência da endoftalmite manifesta é muito inferior a isso. Provavelmente, esse fato deve-se aos mecanismos de defesa intrínseca do olho[8].

A endoftalmite exógena de surgimento tardio ocorre após seis semanas da realização da cirurgia. Geralmente, os patógenos envolvidos são de baixa virulência, como *Propionibacterium acnes*, *S. epidermidis* e, em menor proporção, os fungos[7].

De um modo geral, a incidência de endoftalmite pós-facectomia varia de acordo com o tipo de cirurgia e entre os diferentes estudos, mas tem diminuído ao longo das últimas décadas. No século XIX e no início do século XX, variava entre 5 e 10%[9,10]; 1,5 a 2% na década de 30[10,11]; 0,5 a 0,7% em meados do século passado[10,11]; e 0,06 a 0,09%

no início da década de 90[12,13]. Aprimoramento das técnicas de antissepsia, avanços em materiais cirúrgicos e uso de antibióticos profiláticos, além do melhor conhecimento das causas da infecção, estão entre os principais fatores que podem explicar essa tendência favorável. Nos últimos anos, no entanto, tem sido relatado aumento na incidência de endoftalmite aguda após cirurgia de catarata. Isso coincide com a introdução da técnica de extração da catarata com incisão corneana sem sutura, auto-selante[2,14,15]. West et al. (2005) relataram incidência de 0,2% após cirurgia de catarata entre 1994 e 2001 nos Estados Unidos, com aumento mais significativo nos últimos anos[5]. Nagaki et al. (2003) relataram maior risco com incisão corneana sem sutura (0,29%) em comparação com incisão córneo-escleral (0,05%)[15]. Acredita-se que essa técnica cirúrgica permita pequenos vazamentos quando não completamente selada e, com o piscar dos olhos, a lágrima, que contém bactérias patogênicas, pode ser levada para o interior do olho[16]. Fisiologicamente, esse tipo de incisão fecha devido ao tecido posterior da córnea, que contém uma bomba endotelial de íons. Essa bomba transfere íons e, por osmose, água. Alguma situação que dificulte essa ação fisiológica pode impedir o fechamento adequado da incisão[16]. A variação da pressão intraocular tem sido referida como fator que interfere no risco de endoftalmite. Shingleton et al. (2001) evidenciaram que 21% dos olhos após cirurgia de catarata apresentavam baixos valores de pressão intraocular[17]. Isso favorece ainda mais a entrada de micro-organismos oriundos do meio extraocular.

Considerando que a maioria dos patógenos da endoftalmite exógena provém da microbiota externa[18,19], medidas profiláticas pré-operatórias são muito importantes. O uso de povidona é preconizado como forma de diminuir o risco de infecção[20,21]. O uso de antibióticos tópicos de amplo espectro também foi estudado. Apesar de resultados controversos em alguns estudos, a maioria recomenda seu uso nos períodos pré e pós-operatórios[22].

Após cirurgia filtrante para glaucoma, pode surgir endoftalmite bacteriana aguda ou tardia. A primeira tem incidência variável entre 0,061 e 0,3% e costuma ser causada por *Streptococcus* sp.[7,23,24]. A tardia tem incidência de 0,2 a 18% e é frequentemente causada por *Streptococcus* sp. e *Haemophilus influenza*[7,25].

Endoftalmite também pode ocorrer após vitrectomia via *pars plana*, retinopexia pneumática e ceratoplastia penetrante[26]. No entanto, como são cirurgias menos frequentes que a facectomia, possuem uma representatividade menor na incidência geral.

Após trauma penetrante, a endoftalmite pode ocorrer em aproximadamente 7% dos casos. Demora na sutura, rotura da cápsula do cristalino e ferida suja são fatores de risco independentes para o surgimento da infecção[27]. Os principais micro-organismos causadores da infecção são os cocos gram-positivos e o *Bacillus cereus*[27].

A endoftalmite endógena, representando 2 a 6% de todos os casos de endoftalmite, é causada por fungos em um terço dos casos, especialmente *Candida albicans*[1]. Os outros dois terços são divididos entre bactérias gram-positivas e gram-negativas em proporções semelhantes[28]. Geralmente, está associada a alguma comorbidade sistêmica, como diabetes, cardiopatia e doenças malignas, além de associar-se à presença de outro foco infeccioso[29]. Neste caso, os pacientes podem apresentar abscesso hepático, meningite e endocardite e, subsequentemente, desenvolver endoftalmite endógena[1,6].

Mesmo sendo rara, a endoftalmite é uma infecção potencialmente destrutiva para o olho, que gera dano irreversível à delicada camada de células fotorreceptoras da retina. Até com intervenção terapêutica e cirúrgica, frequentemente resulta em perda parcial ou completa da visão após poucos dias de inoculação[1,18]. Assim, devido o seu caráter grave, é importante o estabelecimento correto e rápido da etiologia da doença para que seja instituída terapêutica apropriada e precoce.

As principais manifestações clínicas da doença, que sempre devem levantar suspeita, são visão embaçada, olho vermelho e dor. Além disso, podem estar presentes edema palpebral, hiperemia conjuntival, quemose, edema corneano, hipópio, vitreíte e hemorragia retiniana difusa[7].

A fim de confirmar o diagnóstico da endoftalmite infecciosa e guiar a escolha do antimicrobiano, deve-se obter material do humor aquoso e do vítreo. O material vítreo tem maior probabilidade de gerar um resultado positivo que o humor aquoso[30]. O vítreo pode ser extraído tanto por punção com agulha fina quanto por vitrectomia. Um trabalho com 138 casos de endoftalmite comprovados por cultura mostrou resultado positivo em 34,8% das punções de câmara anterior, 58,2% das punções vítreas e 80% dos casos submetidos a vitrectomia[31].

O material retirado do meio intraocular é submetido à análise microscópica após coloração por Gram e Giemsa, que mostra células inflamatórias e micro-organismos. Ademais, é semeado em meios de cultura para bactérias e fungos[1]. A cultura de vítreo/aquoso, não obstante ser rotineiramente utilizada como principal exame laboratorial para o diagnóstico das endoftalmites, tem algumas limitações. As duas principais são o longo tempo para a obtenção de um resultado definitivo e a necessidade

de grande quantidade de micro-organismos para o crescimento nos meios de cultura. Em casos clinicamente suspeitos de endoftalmite infecciosa, a cultura mostra uma positividade em torno de 25 a 56% [32-34]. A baixa sensibilidade da cultura microbiológica ocorre em decorrência de vários fatores, como pequena quantidade de amostra, sequestro de micro-organismos em superfícies sólidas (lente intraocular, fragmentos de lentes, cápsula) e consequente diminuição de células no vítreo/aquoso, uso de antimicrobianos antes da coleta do material clínico e presença de micro-organismos fastidiosos como agentes de endoftalmite[34].

O uso da técnica de reação em cadeia de polimerase, PCR, tem aumentado bastante a sensibilidade do diagnóstico da endoftalmite. A PCR amplifica a quantidade de DNA disponível, replicando-o em poucas horas (Figura 1)[35]. Ela é conduzida em três etapas. A primeira, desnaturação, ocorre em altas temperaturas. É necessária para separar a dupla fita de DNA. Na segunda etapa, ocorre o anelamento dos "primers" (oligonucleotídeos iniciadores) na região complementar do DNA molde. A terceira etapa, síntese, é a polimerização através de uma DNA polimerase termoestável (*Taq* DNA polimerase). Com base nessas etapas, várias técnicas de PCR foram desenvolvidas: PCR-RFLP ("restriction fragment length polymorphism"), "PCR-ribotyping", "nested PCR", PCR em tempo real, entre outras[36].

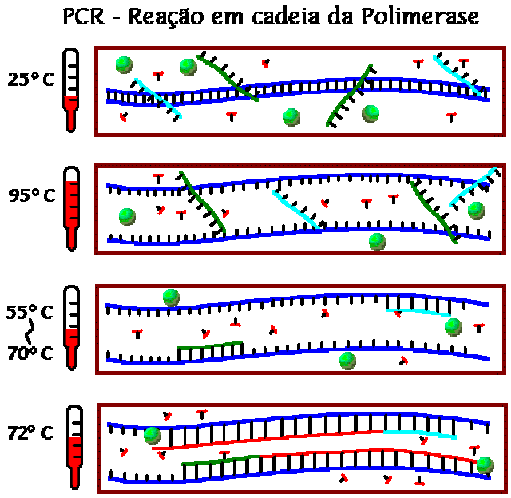

Figura 1- A 25°C, as duas fitas de DNA estão unidas. A 95°C, ocorre a desnaturação, com separação das fitas. Entre 55 e 70°C, os "primers" unem-se a regiões específicas de cada fita de DNA original na fase de anelamento. Por fim, a 72°C, ocorre a formação de uma nova fita de DNA para cada inicial com a ação da DNA polimerase.

Para detectar a presença de algum patógeno bacteriano presente na amostra clínica, "primers" anelando regiões conservadas no genoma de grande parte das bactérias podem ser utilizados[37]. Todas as bactérias compartilham de sequências

comuns de DNA, altamente repetitivas, codificadoras de seu 16S RNA ribossomal. Através do desenho de "primers" para essas regiões conservadas do 16S rDNA, pode ser realizada PCR de material clínico oriundo de olhos com suspeita clínica de endoftalmite e os resultados podem conhecidos em algumas horas[38]. Foi demonstrado que a PCR é capaz de identificar *L. monocytogenes* da câmara anterior do olho três dias antes do resultado definitivo da cultura[39].

As principais vantagens da técnica de PCR são elevadas sensibilidade e capacidade de detectar DNA de um único agente infeccioso (estando ele vivo ou morto), detecção de micro-organismos fastidiosos difíceis ou impossíveis de detectar-se por métodos convencionais de microbiologia e menor tempo necessário para obtenção de resultados[40]. Apesar disso, há algumas desvantagens. Pode haver muitos resultados falso-positivos[33]; o material deve ser cuidadosamente colhido para evitar inclusão de material contaminado; não é rotineiramente disponível devido ao alto custo[41]; e tem uso limitado em infecções mistas[42].

Na oftalmologia, tem sido usada frequentemente para detecção de infecções em situações de risco e em casos em que a cultura é negativa, como nas endoftalmites. Num estudo apresentado em congresso, a PCR foi capaz de identificar micro-organismos em 64% das amostras contra 43% das culturas em amostras de vítreo de casos suspeitos de endoftalmite[43]. Outros estudos mostraram sensibilidade de 92%, 91% e 100% por PCR, contra 24%, 56% e 54%, respectivamente, das culturas[32-34].

Em situações que geralmente apresentam quantidade pequena de micro-organismos, a técnica de "nested PCR" é recomendável para aumentar a sensibilidade diagnóstica[44]. Okhravi et al. (2000), a partir de diluições seriadas contendo micro-organismos viáveis, mostraram que a técnica de "nested PCR" foi capaz de detectar amostras de DNA em tubos com apenas uma célula bacteriana[34]. Essa técnica consiste na amplificação do DNA-alvo em duas etapas e é utilizada quando há uma pequena quantidade de material genético na amostra a ser testada. Dessa forma, para que seja possível a identificação do produto de amplificação do alvo desejado, é feita uma primeira etapa de amplificação com "primers", que irão gerar uma fita de DNA, cuja seqüência-alvo se encontra no produto amplificado. Na segunda fase do processo, "primers" internos são utilizados para amplificar o alvo desejado. Resumidamente, a primeira etapa é aplicada para aumentar a quantidade de DNA contendo a sequência-alvo. Na segunda etapa, o DNA-alvo é amplificado, gerando uma concentração detectável por eletroforese em gel de agarose [34,38,44].

Uma inovadora técnica que está sendo utilizada rotineiramente no diagnóstico de doenças infecciosas é a PCR em tempo real. Possibilita uma nova abordagem diagnóstica, combinando amplificação e quantificação de uma sequência de DNA-alvo através da detecção por sondas específicas marcadas com fluoróforos ou com base na determinação da temperatura de desnaturação de uma sequência de DNA-dupla fita marcadas com substância intercalante fluorescente[45,46].

Os princípios aplicados para detecção de uma seqüência-alvo por PCR em tempo real são baseados na mensuração de fluorescência durante a PCR. A quantidade de produto formada é monitorada durante a reação através da detecção da fluorescência (Figura 2). Esta é proporcional ao produto formado em cada ciclo. O número de ciclos de amplificação ("cycle threshold" – Ct) necessários para obter uma determinada quantidade de DNA é registrado. Com a alta eficiência dos reagentes, sensibilidade dos instrumentos e otimização de técnicas, o número de moléculas de DNA de uma determinada sequência presente em uma amostra pode ser determinado com grande acurácia e sensibilidade[46,47]. Assim, é possível inferir que, quanto menor o número de ciclos em que a pico de fluorescência, maior é a quantidade de DNA na amostra inicial. Quanto maior o Ct, menor é a quantidade inicial do micro-organismo em análise.

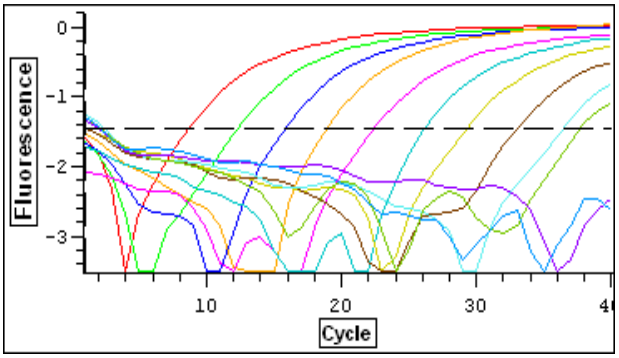

Figura 2- Na PCR em tempo real, a fluorescência emitida é detectada e utilizada para determinar o limiar de ciclo (linha pontilhada) em que a amplificação de DNA entra na fase exponencial. Ou seja, é por desta ferramenta que a técnica fornece resultados quantitativos e permite a comparar a quantidade de DNA na amostra inicial entre diferentes casos de infecção ou mesmo de contaminação.

A detecção de bactérias por PCR em tempo real oferece notáveis benefícios para o paciente. Os resultados podem informar rapidamente o médico sobre a condição infecciosa, permitindo a introdução de antibioticoterapia precoce e mais específica, melhorando o prognóstico, diminuindo a toxicidade dos tratamentos, reduzindo o tempo de hospitalização e prevenindo o uso inapropriado de antibióticos. Isso minimiza o potencial surgimento de linhagens bacterianas resistentes[47]. Um grande benefício da

introdução da PCR em tempo real é aumentar a rapidez com que os resultados podem ser gerados. Isso é possível graças ao tempo reduzido de ciclos de amplificação, à exclusão de etapas pós-PCR e ao uso de equipamento sensível à fluorescência, que permite detecção e monitoramento dos produtos de amplificação simultaneamente[47]. Outra vantagem é a possibilidade de quantificação de DNA, que possibilita diferenciar resultados positivos oriundos de contaminação de resultados em que existe infecção real. Isso é viável por meio da mensuração do número limiar de ciclos em que o micro-organismo é identificado.

A PCR em tempo real possui alta sensibilidade (menos que cinco cópias de uma seqüência-alvo podem ser detectadas) e precisão. Como elimina a necessidade de etapas pós-PCR, evita a possibilidade de contaminação cruzada dos produtos de PCR e reduz o tempo necessário para o processamento da amostra. Existe também a possibilidade de realizar reações múltiplas, o que diminui o tempo para resultados quando se buscam vários alvos. Todos os alvos são amplificados simultaneamente e nas mesmas condições, otimizando a técnica e aumentando a confiabilidade dos dados obtidos[45].

OBJETIVOS

Aspectos epidemiológicos

- Relatar a incidência de endoftalmite bacteriana no Departamento de Oftalmologia da Universidade Federal de São Paulo entre 2002 e 2008

- Identificar os principais micro-organismos causadores de endoftalmite bacteriana e os fatores predisponentes

- Avaliar o perfil de sensibilidade microbiana aos principais antibióticos usados na oftalmologia

Aspectos diagnósticos

- Avaliar a positividade de cultura e bacterioscopia nos casos de endoftalmite bacteriana nas diferentes séries estudadas

- Determinar a aplicabilidade da reação em cadeia de polimerase em tempo real no diagnóstico de endoftalmite bacteriana pós-operatória

- Diferenciar contaminação de infecção por meio do número de ciclos da reação

ARTIGOS

Artigo 1

"Melo GB, Bispo PJM, Regatieri CVS, Yu MCZ, Pignatari ACC, Höfling-Lima AL. Incidence of endophthalmitis after cataract surgery (2002-2008) at a Brazilian university-hospital. Arquivos Brasileiros de Oftalmologia. 2010;73(6):505-7."

Incidence of endophthalmitis after cataract surgery (2002-2008) at a Brazilian university-hospital

Incidência de endoftalmite após cirurgia de catarata (2002-2008) num hospital universitário brasileiro

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Esse estudo teve apoio da FAPESP e da CAPES

ABSTRACT

Purpose: To report on the incidence, diagnostic technique, microbiological features of endophthalmitis at a university-setting in Brazil. **Methods:** All cases of presumed postoperative endophthalmitis from 2002 to 2008 at a teaching-hospital were included. Main data assessed were: number of cataract surgeries performed, incidence of endophthalmitis, microbiological outcome (aqueous and/or vitreous culture and Gram staining), and antimicrobial susceptibility testing of the positive cases. **Results:** Seventy-three eyes of 73 patients (43 females and 30 males) developed endophthalmitis after 24,590 cataract surgeries. The incidence decreased from 0.49% in 2003 to 0.17% in 2006 and stabilized afterwards. Coagulase negative *Staphylococci* (CoNS) and *Streptococcus viridans* (56.5% and 15%, respectively) were the most common bacterial isolates. Culture and Gram stain were negative in 36.9%. CoNS presented susceptibility rates of 80%-sensitivity to oxacillin, 90% to fourth-generation quinolones and 100% to vancomycin. **Conclusions:** The rate of endophthalmitis, diagnostic ability of conventional laboratory investigation, microbial isolates and antibiotic susceptibility are in accordance with other findings of the literature. Despite using prophylactic antibiotic drops, it was possible to identify organisms from infected cases that were susceptible to the antibiotics topically applied.

Keywords: Endophthalmitis incidence, diagnosis, microorganisms, antibiotic susceptibility.

RESUMO

Objetivo: Relatar incidência, técnica diagnóstica e características microbiológicas de endoftalmite numa instituição universitária no Brasil. **Métodos:** Todos os casos de endoftalmite pós-operatória presumida de 2002 a 2008 foram incluídos. Os principais dados avaliados foram: número de cirurgias de catarata realizadas, incidência de endoftalmite, resultado microbiológico (bacterioscopia e cultura de aquoso e vítreo) e teste de sensibilidade antibiótica dos casos positivos. **Resultados:** Setenta e três olhos de 73 pacientes (43 do sexo feminino e 30 do masculino) desenvolveram endoftalmite após 24.590 cirurgias de catarata. A incidência foi reduzida de 0,49% em 2003 para 0,17% em 2006 e estabilizou-se depois disso. *Staphylococcus* coagulase-negativa (SCoN) e *Streptococcus viridans* (56.5% e 15%, respectivamente) foram os isolados bacterianos mais comuns. Cultura e bacterioscopia foram negativas em 36,9%. SCoN apresentou taxas de sensibilidade de 80% à oxacilina, 90% às quinolonas de quarta geração e 100% à vancomicina. **Conclusões:** A taxa de endoftalmite, a capacidade diagnóstica das técnicas laboratoriais convencionais, os micro-organismos isolados e a sensibilidade aos antibióticos estão em acordo com outros achados na literatura. Apesar do uso profilático de colírio antibiótico, foi possível identificar casos de infecção em que as bactérias eram sensíveis aos antibióticos usados topicamente.

Descritores: Incidência de endoftalmite, diagnóstico, micro-organismos, sensibilidade antibiótica.

INTRODUCTION

Infectious endophthalmitis following cataract surgery still is a devastating condition, despite major improvements in surgical techniques in the last decades. Most series report on an incidence rate ranging from 0.05% to 0.4% in different studies worldwide[1].

Most cases are caused by Gram-positive microorganisms present in the conjunctiva and the eyelid. Prophylactic procedures include the use of preoperative and postoperative antibiotics and preoperative povidone. Its management requires a prompt intervention, such as a vitreous tap followed by intravitreal injection of antibiotics or vitrectomy[2].

Herein, we present the rates of endophthalmitis at a university setting in Brazil, where surgeries are predominantly performed by residents and fellows. Additionally, we compare these rates prior and after the introduction of fourth-generation quinolones as postoperative prophylactic drops.

METHODS

This was a retrospective study based on the medical records of the Departments of Ophthalmology and Ocular Microbiology Laboratory (LOFT) at the Federal University of São Paulo, Brazil.

Data from patients who had been previously submitted to cataract surgery (alone or combined with trabeculectomy) were examined and presented with presumed infectious endophthalmitis from 2002 to 2008.

The following data were assessed: number of cataract surgeries performed per year and number of endophthalmitis cases, gender, age, interval from surgery to diagnosis, prophylactic use of antibiotics eye drops, microbiological outcome (aqueous and/or vitreous culture and Gram staining), and antimicrobial susceptibility testing of the positive cases.

The incidence of endophthalmitis was established per year and for the whole period of the study based on its clinical diagnosis. It should be stated that all patients were operated on and followed at the same institution. Patients operated on elsewhere were excluded from this analysis. Antibiotic drops were administered 30 minutes before surgery and for 7 days afterwards (q.i.d.).

The patients were submitted to either vitreous/aqueous tap or vitrectomy followed by intravitreal injection of antibiotics. Intraocular specimens were collected

and cultured on blood agar, chocolate agar, fastidious anaerobic thioglycolate broth, and Sabouraud agar for aerobic and anaerobic bacteria, and fungi. Gram stain and acid-fast stain were performed immediately. A positive culture was defined as either separate colonies of the same organism on two or more separate culture plates or confluent growth at the site of inoculation. Antimicrobial susceptibility testing was performed by the disc diffusion method. Current version of CLSI document M-100, published annually, was used for zone diameter interpretation.

RESULTS

Seventy-three eyes of 73 patients (43 females and 30 males) developed presumed postoperative endophthalmitis after cataract surgery alone (71 cases) or combined with trabeculectomy (2 cases) from 2002 to 2008 at our institution. Demographic data are disclosed in table 1. Most patients were elderly and were under antibiotic drops after surgery until the development of the infection. Mean time interval from surgery to clinical diagnosis was 8.5 days.

In the period of time of this study (2002-2008), 24,590 cataract surgeries were performed at our setting, keeping a regular distribution yearly. Incidence of presumed endophthalmitis varied from a peak of 0.49% in 2003 to a trough of 0.17% in 2006. The rate of endophthalmitis decreased by half when the last 3 years were compared to the 2 initial years. Detailed data are presented in table 2. Overall incidence of endophthalmitis was 0.29%. Microbiological techniques were able to confirm 63% of the clinically suspected cases.

Bacterial species identified are disclosed in table 3. Coagulase negative *Staphylococci* (CoNS) were responsible for 56.5% of all positive cases from 2002 to 2008; *Streptococcus viridans* was positive in 15% of the identified cases; *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* corresponded each to 4% of these cases; other microorganisms were identified in one case each. Among all 73 presumed cases of endophthalmitis, 27 (36.9%) were negative either in culture or in Gram staining.

Antimicrobial susceptibility testing was performed for every positive case of infectious endophthalmitis. None of the Gram-positive isolates were resistant to vancomycin. Five samples of CoNS, and 2 of *Staphylococcus aureus* were resistant to oxacillin. Among these, one sample of CoNS was also resistant to gatifloxacin and another one to moxifloxacin. These patients were under prophylactic drops of

moxifloxacin postoperatively. No other sample of bacteria isolated from these endophthalmitis cases was resistant to the fourth-generation quinolones. It is important to state that during the period of time assessed in this study, antibiotic susceptibility with fourth-generation quinolones was applied to 10 positive samples. Therefore, there was an 80%-sensitivity to this class of antibiotic (90% to gatifloxacin and 90% moxifloxacin each).

DISCUSSION

The incidence of endophthalmitis following cataract surgery has varied over the last decades as described in the literature. In the 1970s, it was 0.32%; 0.16% in the 1980s; 0.08% in the 1990s; and showed a trend to increase in the early years of the 21st century, reaching 0.26%[1]. The increased rate of endophthalmitis after cataract extraction has been temporally associated with the use of sutureless clear cornea incisions. This may be caused by wound defect (including wound leakage), early postoperative hypotony (leading to the inflow of the eyelid microorganisms) and possibly the lack of the conjunctiva covering the corneal incision. The overall rate of endophthalmitis at our setting from 2002-2008 (0.29%) was very similar to the one reported on this systematic review of the literature.

Interestingly, this rate significantly decreased by half from 2002 and 2003 to the following years. Within this period, there was no significant difference in surgical techniques in worldwide phacoemulsification that could explain these data. We hypothesize two possible explanations. First, the phacoemulsification teaching method changed at our institution. Previously, residents that had never done a phacoemulsification would simply begin operating on patients with the assistance from the first to the last step. Later on and nowadays, the residents have to do their first surgeries by performing the last steps, such as corneal suturing and IOL implantation. Only after acquiring some experience, they can perform initial steps and the whole surgery. It is believed that this method might have decreased the rate of surgical complications. However, the impact of the new teaching methodology has not been assessed into details and there are no published data from our service to clearly support this theory. Another possible explanation to a decreased incidence in the latest years is the use of fourth-generation quinolones. They have been routinely used as a postoperative prophylaxis since 2004/2005. And besides, they have been donated to the patients for the postoperative period. Although this association is hardly proven, another

study showed a marked reduction in endophthalmitis incidence with fourth-generation quinolones in comparison to the third-generation ones[3].

One might expect our incidence would be higher than the average since the majority of the cataract surgeries are performed either by residents or fellows. It is well known that surgical complications, such as posterior capsule disruption with vitreous loss, are significant risk factors for the development of this infection. It is also expected that surgeons-in-training be responsible for more cases developing complication[4]. However, a small case series did not show an increased incidence of endophthalmitis after cataract surgery performed by residents[5]. We believe that our results, although presenting an average rate of infection according to the systematic review of literature, is probably higher than it could be due to the higher rate of surgical complications found in surgeries performed by surgeons-in-training.

Laboratory investigation was able to diagnose 63% of the presumed infectious endophthalmitis cases. This is in accordance with most studies, in which culture sensitivity varies from 30 to 80%[6,7]. The sensitivity is increasingly higher with vitreous tap and vitrectomy in comparison to aqueous tap[6].

CoNS are the most common causative microorganisms of infectious endophthalmitis in most series, usually followed by *Streptococci*[8,9]. This was exactly what we found in our study. In the Endophthalmitis Vitrectomy Study, for example, 70% of the causative microorganisms were CoNS and about 9% were *Streptococci* and *Staphylococcus aureus* each[8]. A previous study published by our group also showed a similar distribution of microorganisms regarding all cases of endophthalmitis, including the endogenous and traumatic ones[9].

Despite the lack of clear evidence favoring the use of prophylactic postoperative antibiotics, they are commonly used in most clinical settings. However, this may trigger antibiotic resistance. In two previous studies, it was shown that 68% of CoNS were sensitive to third generation quinolones[10,11]. We showed a 92%-susceptibility to ciprofloxacin in our previous report[9]. In our current series, we found an 80%-susceptibility of CoNS to oxacillin, and a 90%-sensitivity to gatifloxacin or moxifloxacin.

In summary, this study provides information on a large series of endophthalmitis in a single-center from 2002 to 2008. Overall incidence was 0.29% and showed a marked decrease from 2004 on; laboratory investigation was able to diagnose 63% of the presumed infectious cases; CoNS were the most common microorganisms; and there still is a high rate of antibiotic susceptibility in our institution. These data are consistent

with other studies in worldwide literature. One of the main points raised by this report and whose answer remains unknown is how prophylactic antibiotic drops can influence the rate of infectious endophthalmitis. Other important issue is the low sensitivity of the conventional laboratory diagnostic techniques. The clinical application of molecular diagnostic tools should be taken into account. These topics should be addressed in future studies.

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Table 1- Demographic data from the cases (n=73)

Age (mean±SD)		67.6±11.4
Gender	Male	30
	Female	43
Interval (days) between surgery and diagnosis (mean±SD)		8.5±10.11
Prophylactic postoperative use of antibiotic drops	Yes	57
	No	1
	Not available	15

Table 2 - Number of performed cataract surgeries, presumed endophthalmitis cases, culture-proven cases and incidence of endophthalmitis per year and overall rates

Year	Number of surgeries	Presumed endophthalmitis	Culture-proven cases	Incidence
2002	3876	18	11	0.46%
2003	3663	18	13	0.49%
2004	3596	10	5	0.28%
2005	3248	8	5	0.24%
2006	3572	6	4	0.17%
2007	3164	6	4	0.19%
2008	3471	7	4	0.20%
Overall	24590	73	46	0.29%

Table 3 - Microbiological characterization for the whole period of the study (2002-2008). Percentage refers only to the positive cases. NA- not applicable.

Coagulase negative *Staphylococci*	26	56.5%
Streptococcus viridans	7	15.2%
Proteus mirabilis	2	4.3%
Pseudomonas aeruginosa	2	4.3%
Staphylococcus aureus	2	4.3%
Streptococcus pneumoniae	1	2.1%
B-Hemolytic *Streptococcus* (group G)	1	2.1%
Morganella morganii	1	2.1%
Enterococcus spp	1	2.1%
Haemophilus spp	1	2.1%
Acinetobacter	1	2.1%
Weeksella virosa	1	2.1%
Positive Gram staining/negative culture	1	2.1%
Negative culture/Gram staining	27	NA
Total positive cases	46	100%
Total	73	NA

Artigo 2

"Melo GB, Bispo PJM, Yu MCZ, Pignatari ACC, Höfling-Lima AL. Microbial profile and antibiotic susceptibility of culture-positive bacterial endophthalmitis. Eye (Lond). 2011 Feb 18. [Epub ahead of print]"

Observação: artigo selecionado para o Programa de Educação Médica Continuada do Medscape, a pedido do editor da Eye

Microbial profile and antibiotic susceptibility of culture-positive bacterial endophthalmitis

Microbiologic aspects of endophthalmitis

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Abstract

Purpose: To assess the distribution of microorganisms isolated from patients with bacterial endophthalmitis and their antimicrobial susceptibility.

Methods: Retrospective analysis of medical and microbiological records of patients with suspected diagnosis of endophthalmitis. The following information was assessed: number of presumed and culture-positive endophthalmitis cases, source of infection, microbiological result (aqueous and/or vitreous culture and Gram staining), microbial characterization and distribution, and antimicrobial susceptibility.

Results: A hundred and seven (46%) of 231 patients with bacterial endophthalmitis showed positive results by gram-stain or culture. Of these, 97 (42%) patients were positive for culture only. Most of them (62%) were secondary to a surgical procedure (postoperative), 12% were post-traumatic and 26% were secondary to an unknown source or the data were unavailable. A total of 100 microorganisms were isolated (38 aqueous and 67 vitreous samples) from the 97 culture-positive cases (91% were gram-positive and 9% were gram-negative). Coagulase-negative *Staphylococcus* (CoNS) (48%) were the most frequently isolated, followed by *Stretococcus viridans* (18%) and *Staphylococcus aureus* (13%). The antimicrobial susceptibility for CoNS was as follows: amikacin - 91.6%, cephalothin - 97.9%, ceftriaxone - 50%, ciprofloxacin - 62.5%, chloramphenicol - 91.8%, gatifloxacin - 79.5%, gentamicin - 72.9%, moxifloxacin - 89.5%, ofloxacin - 70.8%, oxacillin - 58.3%, penicillin - 33.3%, tobramycin - 85.4%, and vancomycin - 100%.

Conclusion: Gram-positive bacteria were the major causes of infectious endophthalmitis in this large series, usually following surgery. CoNS was the most common isolate. Of interest, susceptibility to oxacillin and fourth-generation quinolones was lower than previously published.

Keywords: Endophthalmitis; Microbial; Antibiotic susceptibility.

Introduction

Infectious endophthalmitis following cataract surgery still is a devastating condition, despite major improvements in surgical techniques in the last decades. Most series report on an incidence rate ranging from 0.05% to 0.4% in different studies worldwide[1].

Most cases are caused by microorganisms from the conjunctiva and the eyelid. They might be secondary to surgery (postoperative endophthalmitis) or to trauma (post-traumatic endophthalmitis). Other microorganisms reach the eye through hematogenic spread (endogenous endophthalmitis)[1-3].

Acute postoperative endophthalmitis is commonly caused by Gram-positive bacteria, especially by coagulase-negative *Staphylococcus* (CoNS), *Staphylococcus aureus*, and *Streptococcus viridans*[3-5]. Low virulence microorganisms, such as *Propionibacterium acnes*, some species of *Streptococci* and fungi, are usually causatives of late postoperative endophthalmitis[6]. Post-traumatic endophthalmitis is caused by the same microorganisms as post-operative cases, as well as other environmental agents[7].

Endogenous endophthalmitis accounts for 2 to 6% of all cases. Previously published causative organisms include *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus agalactiae*, *Clostridium perfringens*, *Moraxella* spp., *Neisseria meningitidis*, *Escherichia coli*, *Klebsiella* spp., *Serratia marcescens*, and *Candida albicans*[3,8].

Its management requires a prompt intervention, such as vitreous tap or vitrectomy followed by intravitreal injection of antibiotics for postoperative and post-traumatic endophthalmitis[9]. Endogenous endophthalmitis requires that the main cause be treated, usually meaning systemic therapy with antibiotics in addition to ocular therapy.

Taking this into account, this study aimed at assessing the distribution of microorganisms isolated from patients with bacterial endophthalmitis and their antimicrobial susceptibility from 2006 to 2009 and compare them to the data previously published by our group (2000-2005)[3].

Materials and Methods

This was a retrospective study based on medical records from the Departments of Ophthalmology and Ocular Microbiology Laboratory at the Federal University of São Paulo, Brazil.

Data from all patients with presumed infectious endophthalmitis from January, 1st 2006 to October, 31st 2009 were analyzed. Presumption of endophthalmitis was based on a well-characterized clinical diagnosis.

The following information was assessed: number of presumed and culture-positive endophthalmitis cases, source of infection (postoperative, post-traumatic, endogenous and unknown), microbiological result (aqueous and/or vitreous culture and Gram staining), microbial characterization and distribution, and antimicrobial susceptibility testing of the positive cases for amikacin, cephalothin, ceftriaxone, ciprofloxacin, chloramphenicol, gatifloxacin, gentamicin, moxifloxacin, ofloxacin, oxacillin, penicillin, tobramycin, and vancomycin.

The patients were submitted either to vitreous/aqueous tap or vitrectomy followed by intravitreal injection of antibiotics. Intraocular specimens were collected and cultured on blood agar, chocolate agar, fastidious anaerobic thioglycolate broth, and Sabouraud agar for aerobic and anaerobic bacteria, and fungi. Gram stain and acid-fast stain tests were performed immediately. A positive culture was defined as either separate colonies of the same organism on two or more separate culture plates or confluent growth at the site of inoculation. Antimicrobial susceptibility testing was performed by the disc diffusion method. Current version of CLSI document M-100, published annually, was used for zone diameter interpretation.

Results

Aqueous and vitreous samples from 231 patients with presumed endophthalmitis of different origins were sent to our laboratory of ophthalmic microbiology throughout the period of this study. A hundred and seven (46%) of 231 patients with bacterial endophthalmitis showed positive results by gram-stain or culture. Of these, 97 (42%) patients were positive for culture only. Smears were positive for bacteria in 10 culture-negative and in 47 culture-positive cases. Culture was positive in 41 samples obtained from the aqueous humor, 34 from vitreous tap and 33 from vitrectomy samples. There was overlap among them in 15 cases.

A total of 100 microorganisms were isolated (38 from aqueous and 67 from vitreous samples) from the 97 culture-positive cases (91% were gram-positive and 9%

were gram-negative). Table 1 shows detailed microbial distribution. Coagulase-negative *Staphylococcus* (CoNS) (48%) were the most frequently isolated, followed by *Streptococcus viridans* (18%) and *Staphylococcus aureus* (13%). Three cases were diagnosed with 2 microorganisms. CoNS was identified in each of them. Contamination from the lid microbiota might have happened.

Most culture positive cases (62%) were secondary to a surgical procedure (postoperative), 12% were post-traumatic and 26% were secondary to an unknown source or the data were unavailable (Table 2). CoNS was the most common microorganism regardless the predisposing factor. Of note, the 2 cases caused by *Bacillus sp.* were secondary to trauma.

Antibiotic susceptibility for the 5 more prevalent groups of microorganisms is disclosed in Table 3. It should be pointed out that 33% of the cases of CoNS resistant to gatifloxacin were sensitive to oxacillin (methicillin-sensitive) and 67% were also resistant to oxacillin (methicillin-resistant). All CoNS moxifloxacin-resistant cases were also methicillin-resistant.

Discussion

In order to achieve effective prophylaxis and treatment of endophthalmitis, it is important to know the most common etiologic agents involved and their response to the major classes of antibiotics routinely used.

The data in this study are quite similar to those from our previous report (155 positive samples). From 2000 to 2005, CoNS (42%), *Streptococcus viridans* (14%) and *Staphylococcus aureus* (8%) prevailed. Postoperative infection was, as well as in this series, the most prevalent source (61%). Regarding antibiotic susceptibility, CoNS was 83%-sensitive to oxacillin and 100%-sensitive to the fourth-generation quinolones and vancomycin[3].

In the present series of 231 infectious endophthalmitis cases, 46.3% were found to have a positive gram-stain or culture result. The remaining cases did not have a conclusive microbiological culture. The low sensitivity of this technique may be due to different factors, such as small amount of available microorganisms or previous use of antibiotics[10]. Molecular biology techniques, especially polymerase chain reaction, disclose sensitivities from 90 to 100% in most studies[11,12].

Culture and Gram staining positivity were similarly distributed among aqueous, vitreous tap and vitrectomy specimens. Fifteen percent were simultaneously identified

from 2 different sources (aqueous and vitreous tap or vitrectomy samples). These data were different from our previous study in which vitreous tap specimens made up 51% of all positive cases. Since clinical data, such as visual acuity, were not available for this analysis, this difference could simply be explained by a different severity at presentation. Assuming this hypothesis, vitrectomy might have been more used in the present series due to more severe cases and, therefore, disclosed more positive cases than previously.

Gram-positive bacteria were responsible for 91% of culture-positive cases whereas gram-negative caused 9% of them. Most published articles show a predominance of gram-positive microorganisms, ranging from 63 to 86%[13-15]. In a previous report from our laboratory (2000-2005), gram-positive bacteria comprised 79% of the culture-proven endophthalmitis isolates[3]. In our present and previous analyses, CoNS, *Streptococcus viridans*, and *Staphylococcus aureus* were the three most common bacteria in a descending order. This is in agreement with the most common microorganims in the conjunctival flora[16].

As expected, most cases were secondary to a surgical procedure (62%) and to trauma (12%). The microorganisms profile in the postoperative and post-traumatic groups is that present in conjunctival flora in both and from the environment in the latter. It should be remarked that *Bacillus sp.* was only present in the post-traumatic group.

The most relevant information of this study refers to the antimicrobial susceptibility. In our previous report, comprising data from 2000 to 2005, all CoNS were susceptible to both moxifloxacin and gatifloxacin[3]. From 2006 to 2009, 79.5% and 89.5% of CoNS were sensitive to gatifloxacin and moxifloxacin, respectively. Other studies in the literature have shown this rate to vary from 65 to 96%[17,18]. Additionally, most fourth-generation quinolone-resistant samples were also found to be methicillin-resistant in our study. A recently published article showed that most methicillin-resistant samples from the conjunctiva were also quinolone-resistant[19]. Betanzos-Cabrera et al. (2009) have just shown how this resistance mechanism occurs. All strains of CoNS resistant to quinolones (gatifloxacin and moxifloxacin) presented mutations at Ser84Phe for the gyrA gene, and Ser80Phe for the parC gene in their series[20].

Since the fourth-generation quinolones are routinely used in the prophylaxis of many surgical procedures, this increasing trend of CoNS resistance is of concern. Besides that, due to their vitreous penetration above MIC, oral moxifloxacin is used in some settings as an additional treatment of endophthalmitis.

The fourth-generation quinolones showed good in vitro results against *Staphylococcus aureus, Streptococci* and *Pseudomonas*. All gram-positive microorganisms were susceptible to vancomycin.

In summary, gram-positive bacteria were the major causes of infectious endophthalmitis in this large series, usually following surgery. CoNS was the most common isolate. Of interest, susceptibility to oxacillin and fourth-generation quinolones was lower than previously published.

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Table 1- Microbial distribution by species

Isolated microorganisms	Number of isolates
CoNS	48
Streptococcus viridans	18
Staphylococcus aureus	13
Pseudomonas aeruginosa	5
Streptococcus pneumoniae	3
Corynebacterium sp	3
Other Streptococci	2
Gram-positive Bacillus	2
Moraxella nonliquefaciens	2
Serratia sp	1
Propionibacterium acnes	1
Nocardia sp	1
Weeksella virosa	1
Total	100

Table 2- Microbial distribution according to the source of the infection

Isolated microorganisms	Postoperative	Post-traumatic	Endogenous	Unknown/Unavailable
CoNS	32	4		12
Streptococcus viridans	14	1		3
Staphylococcus aureus	5	3		5
Pseudomonas aeruginosa	2			3
Streptococcus pneumoniae	2	1		
Corynebacterium sp	1	1		1
Other Streptococci	1			1
Gram-positive Bacillus		2		
Moraxella nonliquefaciens	2			
Serratia sp	1			
Propionibacterium acnes	1			
Nocardia sp				1
Weeksella virosa	1			
Total	62	12	0	26

Table 3- Antimicrobial susceptibility to the most frequent isolated microorganisms

	CoNS		*Staphylococcus aureus*		*Streptococcus viridans*		*Streptococcus pneumoniae*		*Pseudomonas aeruginosa*	
	N	%	n	%	n	%	n	%	n	%
Amikacin	48	91.6	13	84.6	-	-	-	-	5	100
Cephalothin	48	97.9	13	69.2	18	94.4	3	100		
Ceftriaxone	48	50	13	69.2	18	83.3	3	100	5	0
Ciprofloxacin	48	62.5	13	92.3	18	50	3	100	5	100
Chloramphenicol	48	91.8	12	100	18	94.4	3	100	5	20
Gatifloxacin	44	79.5	11	100	14	92.8	2	100	5	100
Gentamicin	48	72.9	12	100	-	-	-	-	5	100
Moxifloxacin	48	89.5	12	91.6	13	100	3	100	5	100
Ofloxacin	48	70.8	12	100	18	100	3	100	5	100
Oxacillin	48	58.3	12	75	18	50	3	66.6	-	-
Penicillin	48	33.3	12	8.3	18	94.4	3	100	-	-
Tobramycin	48	85.4	12	83.3	-	-	-	-	5	100
Vancomycin	48	100	13	100	18	100	3	100	-	-

n- total number of samples assessed; %- percentage of susceptibility

Artigo 3

"Melo GB, Bispo PJM, Pignatari ACC, Höfling-Lima AL. Real-Time PCR Test to Discriminate between Contamination and Intraocular Infection after Cataract Surgery. Aceito para publicação no Journal of Cataract and Refractive Surgery"

Carta de aceite:

De: Journal of Cataract and Refractive Surgery <jcrs@ASCRS.org>

Para: gustavobmelo@yahoo.com.br

Enviadas: Sexta-feira, 14 de Janeiro de 2011 12:49:00

Assunto: Your Submission, "Real-Time PCR Test to Discriminate between Contamination and Intraocular Infection after Cataract Surgery"

Ref.: Ms. No. JCRS-10-1066R1

Real-Time PCR Test to Discriminate between Contamination and Intraocular Infection after Cataract Surgery

Journal of Cataract & Refractive Surgery

Dear Dr. Melo,

The reviewers and editor have accepted your (revised) manuscript "Real-Time PCR Test to Discriminate between Contamination and Intraocular Infection after Cataract Surgery" (JCRS-10-1066R1) for publication in the journal. As soon as it is scheduled for a particular issue, you will receive typeset pages to check.

Sincerely,

Nick Mamalis, MD

Editor

Journal of Cataract & Refractive Surgery

Real-Time PCR Test to Discriminate between Contamination and Intraocular Infection after Cataract Surgery

Real-Time PCR after Cataract Surgery

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Abstract

Purpose: To determine the usefulness of real-time polymerase chain reaction (PCR) assays in the diagnosis of post-operative bacterial endophthalmitis in clinically diagnosed infectious cases and to test for bacterial DNA in control samples collected from non-infected eyes.

Setting: Federal University of São Paulo, Brazil.

Methods: Eleven patients with clinically diagnosed infectious endophthalmitis (9 vitreous and 7 aqueous samples) after cataract surgery were included, as well as 12 vitreous (samples from non-inflamed eyes obtained through vitrectomy) and 50 aqueous samples (at the end of phacoemulsification) from control subjects at a single-university setting. Universal and Gram-specific real-time PCR, Gram staining, and culture were carried out. Sensitivity and cycle thresholds (Ct) were determined. Clinical and microbiological data were also assessed.

Results: Gram and culture were able to identify 80% and 75%, respectively, of the patients with infectious endophthalmitis. Real-time PCR assays were positive in 91% of patients with a clinical diagnosis of endophthalmitis, using either aqueous and/or vitreous samples. None of the 12 vitreous controls were positive by PCR. Two of the aqueous control samples were positive by real-time PCR. The Ct cutoff value for universal-PCR was 36 (sensitivity: 93.8%; specificity: 100%) and 38 for Gram-specific-PCR (sensitivity: 93.8%; specificity: 100%). Gram-positive microorganisms prevailed and visual acuity varied according to the causative bacteria.

Conclusions: Real-time PCR is a fast and accurate diagnostic tool in cases of bacterial endophthalmitis. As a quantitative technique, it may also serve as a new and unique application: the distinction between contamination and infection based on the Ct value.

INTRODUCTION

Infectious endophthalmitis following cataract surgery is still a devastating condition, despite major improvements in surgical techniques in the last decades. Most series report an incidence rate ranging from 0.05% to 0.4% in different studies worldwide[1].

Most cases are caused by Gram-positive microorganisms present in the conjunctiva and the eyelid. Their management requires a prompt intervention, such as a vitreous tap followed by intravitreal injection of antibiotics or pars plana vitrectomy (PPV)[2].

Diagnosis may be achieved with conventional microbiological techniques, such as Gram staining and culture. However, they are time-consuming and have low sensitivity. Additionally, it is not possible to tell through microbiological techniques whether a positive result represents a true infectious disease or just post-operative contamination.

In order to improve sensitivity along with increased speed, polymerase chain reaction (PCR) has been increasingly used for this purpose. Real-time PCR technology is an enhancement of the original PCR design. It is a homogeneous method in which DNA amplification and detection of the target sequence occur together, decreasing handling of PCR products and risks of carryover contamination.[3] Real-time PCR protocols to detect bacterial DNA in clinical samples of aqueous and vitreous humor have been recently described[4,5]. Our previous study demonstrated that real-time PCR is a fast and sensitive method for the laboratory characterization of endophthalmitis, with the whole reaction lasting less than 5 hours[6]. Real-time PCR amplifies a specific target sequence in a sample and then monitors the amplification progress using fluorescent technology. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of original target sequence, thereby enabling quantification. Amplification curves are graphed by the software to help determine the cycle threshold (Ct) at which fluorescence reaches a threshold level. The higher the number of Ct, the lower the amount of target sequence is[7].

Therefore, the purpose of this study was to determine the usefulness of a real-time PCR assay to diagnose post-phacoemulsification endophthalmitis cases and to set a

Ct cutoff to discriminate between post-operative contamination and true endophthalmitis in clinically diagnosed infectious cases.

METHODS

Clinical Samples from Infected Eyes

This was a prospective study that included 11 patients with a clinical diagnosis of infectious post-phacoemulsification endophthalmitis seen at our clinical setting between November 2007 and December 2009.

The diagnosis of endophthalmitis was made on the basis of clinical features, including decreased vision, periocular pain, and anterior and posterior segment inflammation. All patients had vitreous infiltration diagnosed by clinical examination or ophthalmic ocular ultrasound.

Aqueous and vitreous humor samples were obtained by means of anterior chamber paracentesis and vitreous tap, respectively. Vitreous samples were also collected by vitrectomy through the *pars plana*. An aliquot of the samples, which could be diluted or undiluted, was immediately used for Gram and Giemsa staining and cultured in blood agar, chocolate agar, and thioglycolate broth under aerobic and anaerobic conditions for 14 days. Bacteria were identified using manual biochemistry-based tests and, when necessary, the Phoenix automated system (BD Diagnostic System, Sparks, MD).

Around 100 to 500 μl of the samples were aseptically transferred to a sterile microtube and stored at -20°C for PCR procedures. After vitreous tap or vitrectomy, all eyes were treated with intravitreal injection of vancomycin (1 mg) and ceftazidime (2.25 mg).

The Declaration of Helsinki guidelines were followed. The study was IRB approved, and informed consent was obtained from each patient.

Control Samples from Non-Infected Eyes

Fifty different aqueous samples obtained at the end of routine phacoemulsification were included as controls to establish the rates of anterior chamber contamination and the Ct cutoff value of our real-time PCR assays for laboratory diagnosis of endophthalmitis in this set of patients. Moreover, 12 vitreous samples were obtained from 12 eyes without intraocular inflammatory/infectious diseases at the moment of vitrectomy for different therapeutic purposes, such as rhegmatogenous

retinal detachment, macular hole and epiretinal membrane. None of these patients developed endophthalmitis. The samples were aseptically transferred to a sterile microtube and stored at -20°C for PCR processing.

DNA Extraction from Aqueous and Vitreous Samples

Total DNA was extracted from aqueous and vitreous humor using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the tissue protocol with few modifications. In brief, 50 µl of sample were mixed with 180 µl tissue lysis buffer (ATL) and immediately ground in liquid nitrogen for 1 minute. Extraction was achieved after treatment with proteinase K (10 minutes incubation at 56°C) and lysis buffer (AL) (10 minutes incubation at 70°C). After spin purification, DNA was eluted with 100 µl elution buffer (EB) and stored at -20°C.

Prevention of Contaminant DNA Amplification

PCR mixtures were pretreated with DNaseI to eliminate background DNA contamination as previously reported[6]. Sample extraction and PCR preparation were carried out using a laminar hood and DNA workstation, respectively, with pipettes and plugged tips in a pre-PCR room.

Real-Time PCR Assays

Universal detection of bacterial DNA from aqueous and vitreous samples was done using a SYBR Green 16S rDNA-Based Universal PCR (SGRU-PCR) and Gram discrimination by a Multiplex Gram-Specific TaqMan-Based PCR (MGST-PCR) as previously described[6]. Both reactions were performed with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), using 2 µl of purified DNA extracted from aqueous and vitreous samples, in a 20-µl reaction volume. Briefly, SGRU-PCR was prepared containing 10 µl of Platinum SYBR Green qPCR Super Mix (Platinum *Taq* DNA polymerase, SYBR Green I dye, Tris-HCl, KCl, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP, 800 µM dUTP, DNA uracil glycosylase and stabilizers), pretreated with 0.25 U RQ1 RNase-free DNase I (Promega, Madison, USA) and 0.2 µM of each primer, NT-341Fw and 16S-522Rv. Amplification was performed with the following conditions: 50°C for 2 minutes and 95°C for 10 minutes (UDG) followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Melting curve analysis was done after amplification by heating samples from 68°C to 95°C with increments of 0.5°C/s. The MGST reaction consisted of 10 µl TaqMan Universal PCR Master Mix,

pretreated with 0.4 U of DNaseI, 0.25 μM of each primer, NT-341Fw and 16S-522Rv, 0.15 μM GN-Probe and 0.05 μM GP-Probe (Applied Biosystems, Foster City, CA). Amplification was carried out under the following conditions: 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Cycle threshold (Ct) is determined after reaction by setting the threshold line in the linear phase of the amplification plot. The intersection of the amplification curve and threshold line is determined as the Ct value by the thermocycler software. Amplification of β-globin gene was carried out for every clinical sample at the same time of bacterial determination. The amplification of every 5 clinical samples was done in the presence of a negative control including all PCR reagents except DNA template and positive controls using 10 ng/μl *S. epidermidis* and *E. coli* DNA. Primers are displayed in Table 1.

Receiver Operating Characteristic Curve Analysis

ROC curve analysis was performed using SPSS version 17.0 (SPSS, Inc., Chicago) in order to assess the accuracy of the tests and to determine a cutoff value for each real-time PCR assay. To set the Ct cutoff value for SGRU-PCR, all samples from patients with a clinical diagnosis of infectious endophthalmitis were considered. For MGST-PCR, the Ct values recorded by the GP and GN probes for sample matching with microscopy and culture were used. Sensitivity and specificity were calculated for each cutoff point. Sensitivity values for each Ct point were plotted against the corresponding values of 1-specificity, resulting in a ROC curve. The area under curve (AUC) was determined for each real-time PCR assay.

RESULTS

Nine vitreous and 7 aqueous samples were obtained from the 11 patients with clinical diagnosis of post-operative infectious endophthalmitis. Two of the patients only had an aqueous sample available for analysis.

The mean age of the patients with endophthalmitis was 66.6 years. Six were males (55%) and 5 were females (45%). All cases but one were acute and under concomitant use of prophylactic antibiotic drops. Detailed data are disclosed in Tables 2 and 3.

Gram staining was positive in 78% of the vitreous samples and in 80% of the aqueous samples. Culture was positive in 78% of the vitreous samples and in 71% of

the aqueous ones. Real-time PCR assays were positive in 89% of the vitreous samples and in 100% of the aqueous samples. Overall, it was possible to diagnose infectious endophthalmitis in 91% of the patients tested with this technique (Table 4).

Gram-positive microorganisms were the most causative pathogens in this case series. The most commonly identified bacteria were *Streptococcus* spp. (n=6) followed by coagulase-negative *Staphylococcus* (n=3). *Propionibacterium acnes* was identified in 1 sample. Gram negative bacilli were identified in 4 samples, 2 *Acinetobacter* spp. and 2 *Flavobacteriaceae* isolates.

Although no statistical analysis was performed due to the small subgroup sample size, we observed that final outcome was not influenced by the bacterial DNA load. The best final visual acuity was achieved for patients with culture negative samples and those positive for Gram-negative and CoNS pathogens. Eyes infected by streptococci displayed worse final visual acuity regardless of DNA load and type of treatment.

There was a good correlation between Gram stain, culture and Multiplex PCR for Gram classification.

None of the 12 control vitreous samples were positive for bacteria by real-time PCR. Control samples from the anterior chamber at the end of cataract surgeries disclosed a contamination rate of 4% by SGRU-PCR and 0% by MGST-PCR. None of them developed endophthalmitis. Ct values for the 2 positive control samples were 39. According to the ROC curve analysis (Table 5), considering a Ct range for infected and control samples, the best Ct cutoff for SGRU-PCR was 36 (sensitivity: 93.8%; specificity: 100%) and 38 for MGST-PCR (sensitivity: 93.8%; specificity: 100%).

The microorganisms and their susceptibility are shown in Table 6. *Streptococcus sp.* prevailed (4 in 8 patients; 50%), followed by S*taphylococci* (2 out of 8 - 25%). Gram-positive bacteria comprised 75% of those identified by culture. It is important to mention that one CoNS was resistant to both oxacillin and moxifloxacin.

DISCUSSION

A prompt and accurate diagnosis of endophthalmitis is required in order to establish a correct therapy. In most cases, broad-spectrum antibiotics are used as the first step associated with either vitreous tap or vitrectomy. Vitreous samples are usually submitted to conventional microbiological analysis in order to identify the causative organisms. However, Gram staining and culture have sensitivities ranging from 6 to

54%, depending on the moment and source of sampling[8-10]. PCR has been shown to increase this diagnostic rate to as high as 92%[5,11]. Since antibiotic injection is usually primarily performed, the most important application of PCR, especially real-time PCR, would be a) the rapid detection of different microorganisms not covered by the antibiotics and b) differentiation between infection and contamination and reassessment of diagnosis and therapeutic options after an unresponsive first treatment.

We were able to show an increased rate of microbial detection in bacterial endophthalmitis cases with our previously standardized universal (SYBR Green) and Multiplex (Gram-positive and Gram-negative) PCR techniques to 100 and 90% (aqueous humor and vitreous).

Considering only the results of the universal PCR, all Ct values in bacterial endophthalmitis cases ranged between 19.5 and 34.5. The 2 contaminated samples from the aqueous humor displayed a Ct of 39. This difference allows us to establish a cutoff value to distinguish between infection and contamination. Although limited by the small number of contaminated aqueous samples, this finding may add an important application of real-time PCR in the diagnosis of post-phacoemulsification endophthalmitis. It is also important to remark that these Ct values are applicable to this PCR methodology. Different methods require different standardization and show different cutoff data.

Sugita et al (2010) have recently published the first study to use quantitative PCR to diagnose infectious endophthalmitis[12]. Using a similar sample size as ours, they were able to quantify the amount of bacteria in samples from infectious cases, but none of their controls were positive.

Microbial distribution here differs from that of other series, even though Gram-positive bacteria still comprised the majority of the culture isolates. In our series, *Streptococci* were the most common microorganisms isolated in culture. Although *Staphylococci* are usually more frequent, other studies using molecular techniques also disclosed a high frequency of these microorganisms in ocular infections [8-10,13].

Some studies have also shown that bacteria may be found in the anterior chamber at the end of cataract surgery without leading to endophthalmitis[14,15]. This could be a problem in the application of conventional PCR techniques for this diagnosis. The possibility of using a cutoff point raises a new perspective in order to discriminate between contamination and infection.

When considering antibiotic susceptibility, it is of note that one of the methicillin-resistant CoNS was also resistant to moxifloxacin, which agrees with recent

reports from the literature[16,17]. Therefore, this finding supports the increased trend for CoNS resistance to oxacillin (methicillin) and fourth-generation quinolones.

It should be pointed out that a very uncommon microorganism was identified, namely *Weeksella virosa*. There are a few reports of this Gram-negative bacillus in the urinary tract and as a cause of peritonitis[18,19]. In our study, it caused acute endophthalmitis 5 days after PPV for the removal of a crystalline lens from a phacoemulsification 10 days earlier. The 50-year-old man had no associated systemic or other ocular diseases. Treatment with intravitreal antibiotics resolved the infection, and final visual acuity was up to 20/60 after one month.

We can conclude that real-time PCR is a fast and accurate tool for the diagnosis of bacterial endophthalmitis. As PCR is also a quantitative technique, it serves as a new and unique application: distinction between contamination and infection based on Ct values. However, conventional techniques still have a role, especially due to the easy assessment of antibiotic susceptibility.

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Table 1- Primers and probes used in this study

	Sequence (5'- 3')	Application	Product size (bp)
Primers			
NT-341Fw	GACTCCTACGGGAGGC	16S rDNA Universal Amplification	192
16S-522RV	GCGGCTGCTGGCAC	16S rDNA Universal Amplification	192
Beta-Rv	CCAAGAGTCTTCTCTGTCTC	Endogenous Control	100
Beta-Fw	GAAGTTGGTGGTGAGGCC	Endogenous Control	100
Probes			
GP-Probe	FAM - CTGAYSSAGCAACGCCGCG - MGB	Gram-positive Classification	--
GN-Probe	VIC - CCTGAYSCAGCMATGCCGCG - MGB	Gram-negative Classification	--
Beta-Probe	NED - AGGTTGGTATCAAGGTTACAAG - MGB	Endogenous Control	--

Table 2- Demographic data and ocular history of the patients

Patient	Age	Ocular history	Initial VA	Final VA	Treatment	Prior antibiotics
1	69	Phacoemulsification 1 day ago	LP	LP	Vitreous tap	Yes
2	86	Phacoemulsification 1 week ago	LP	HM	Vitreous tap	Yes
3	57	Phacoemulsification 10 months ago	LP	NA	PPV	No
4	66	Phacoemulsification 5 days ago (prior PPV)	HM	20/200	Vitreous tap	Yes
5	50	Phacoemulsification 15 days ago and PPV 5 days ago	HM	20/60	Vitreous tap	Yes
6	72	Phacoemulsification+trabeculectomy 4 days ago	LP	20/100	PPV	Yes
7	77	Phacoemulsification 1 week ago	LP	NA	PPV	Yes
8	65	Phacoemulsification 1 day ago	LP	LP	PPV	Yes
9	60	Phacoemulsification 5 days ago	HM	20/70	PPV	Yes
10	65	Phacoemulsification 1 day ago	HM	20/200	Vitreous tap	Yes
11	66	Phacoemulsification 1 day ago	LP	NA	Vitreous tap	Yes

*Abbreviations: PPV - pars plana vitrectomy; IOL- intraocular lens; HM- hand motion; NLP- no light perception; LP- light perception; CF: counting finger; NA- not available.

Table 3- Conventional microbiology and PCR results for each of the patients/samples

Patient	Sample	Gram staining	Culture	Universal PCR	GP PCR	GN PCR	Sequencing
1	Vitreous	Gram positive cocci	*Streptococcus viridans*	20.5	25.0	-	*Streptococcus mitis* group
	Aqueous	Gram positive cocci	*Streptococcus viridans*	26.7	33.8	-	*Streptococcus mitis* group
2	Vitreous	Gram positive cocci	*Beta-hemolytic Streptococcus*	18.7	20.9	-	*Streptococcus dysgalactiae*
3	Vitreous	-	*Propionibacterium acnes*	33.6	30.0	34.9	*P. acnes*, Actinobacterium spp., Mycobacterium spp.
4	Vitreous	Gram positive cocci	-	20.9	23.9	-	Coagulase-Negative *Staphylococci*
	Aqueous	Gram positive cocci	-	26.5	30.2	29.2	Coagulase-Negative *Staphylococci*
5	Vitreous	Gram negative bacilli	*Weeksella virosa*	24.4	-	23.8	*Flavobacteriaceae*
	Aqueous	Gram negative bacilli	*Weeksella virosa*	24.6	-	23.5	*Flavobacteriaceae*
6	Vitreous	Gram positive cocci	*CoNS*	21.2	29.0	-	Coagulase-Negative *Staphylococci*
7	Vitreous	Gram negative bacilli	*Acinetobacter calcoaceticus*	22.0	-	20.6	*Acinetobacter* spp.
	Aqueous	-	*Acinetobacter calcoaceticus*	23.6	-	21.9	*Acinetobacter* spp.
8	Vitreous	Gram positive cocci	*Streptococcus viridans/Staphylococcus aureus*	28.6	28.2	-	*Streptococcus mitis* group
	Aqueous	NA	*Streptococcus viridans/Staphylococcus aureus*	26.1	35.0	-	*Streptococcus mitis* group[a]
9	Vitreous	-	-	-	-	-	NA
10	Aqueous	NA	-	32.5	-	34.8	Bad File (Mixed Sequence)
11	Aqueous	Gram positive cocci	*Streptococcus pneumoniae*	19.5	23.2	-	*S. pneumoniae* and *S. mitis* group

* Abbreviations: NA- not available; CoNS- coagulase-negative *staphylococcus*; PCR- polymerase chain reaction; GP- gram-positive; GN- gram-negative

Table 4- Sensitivity of each technique according to the source of the sample

Sensitivity (%)			
	Aqueous	Vitreous	Overall
Gram staining	80	78	80
Culture	71	78	73
PCR	100	89	91

* Abbreviation: PCR - polymerase chain reaction

Table 5- Results of Sensitivity and Specificity for each Ct point tested by the ROC curve analysis

SGRU-PCR (AUC = 0.968)			MGST-PCR (AUC = 0.968)		
Ct cutoff value	Sensitivity (%)	Specificity (%)	Ct cutotf value	Sensitivity (%)	Specificity (%)
17.7	0	100	20.0	0	100
19.1	6.3	100	21.5	12.5	100
20.0	12.5	100	22.5	18.8	100
20.7	18.8	100	23.5	25.0	100
21.0	25.0	100	24.5	43.8	100
21.6	31.3	100	26.5	50.0	100
22.8	37.5	100	28.5	56.3	100
24.0	43.8	100	29.5	62.5	100
24.5	50.0	100	32.0	75.0	100
25.3	56.3	100	34.5	81.3	100
26.3	62.5	100	38.0 *	93.8	100
26.6	68.8	100	42.0	100	0
27.6	75.0	100			
30.5	81.3	100			
33.0	87.5	100			
36.3 *	93.8	100			
40.0	93.8	68			
42.0	100	0			

* The best Ct cutoff value that optimize sensitivity and specificity; AUC = Area Under Curve

Table 6- Antimicrobial susceptibility to oxacillin, vancomycin, moxifloxacin and amikacin for the samples identified by culture.

Patient	Sample	Microorganism	Oxacillin	Vancomycin	Moxifloxacin	Amikacin
1	Vitreous	*Streptococcus viridans*	NS	S	S	NS
	Aqueous	*Streptococcus viridans*	NS	S	S	NS
2	Vitreous	*Beta-hemolytic Streptococcus*	S	S	S	NS
3	Vitreous	*Propionibacterium acnes*	NA	NA	NA	NA
5	Vitreous	*Weeksella virosa*	NA	NA	NA	NA
	Aqueous	*Weeksella virosa*	NA	NA	NA	NA
6	Vitreous	*CoNS*	NS	S	NS	S
7	Vitreous	*Acinetobacter calcoaceticus*	NS	NS	S	S
	Aqueous	*Acinetobacter calcoaceticus*	NS	NS	S	S
8	Vitreous	*Streptococcus viridans*	S	S	S	NS
	Vitreous	*Staphylococcus aureus*	S	S	S	S
11	Aqueous	*Streptococcus pneumoniae*	S	S	S	NS

* Abbreviations: CoNS- coagulase-negative *staphylococcus;* NS- not susceptible; S- susceptible; NA- not available

CONCLUSÕES

Aspectos epidemiológicos

- A incidência global de endoftalmite foi de 0,29% e apresentou redução significativa ao longo do período de análise: de 0,46% em 2002 para 0,20% em 2008.

- Cocos gram-positivos foram os principais causadores de endoftalmite bacteriana. *Staphylococcus* coagulase-negativa e *Streptococcus viridans*, respectivamente, foram os mais frequentes entre eles. A maioria dos casos foi ocorreu após procedimentos cirúrgicos

- Os micro-organismos mais prevalentes foram sensíveis à maioria dos antibióticos usados rotineiramente na oftalmologia. No entanto, houve maior resistência dos *Staphylococcus* coagulase-negativa à oxacilina e às quinolonas de quarta geração, de acordo com o artigo 2, do que previamente publicado na literatura

Aspectos diagnósticos

- A positividade das técnicas convencionais (bacterioscopia e cultura) variou de 43 a 80% nas diferentes séries analisadas nesta tese

- A reação em cadeia de polimerase em tempo real mostrou-se uma ferramenta com boa aplicabilidade prática e apresentou sensibilidade global de 91% nos casos de endoftalmite bacteriana

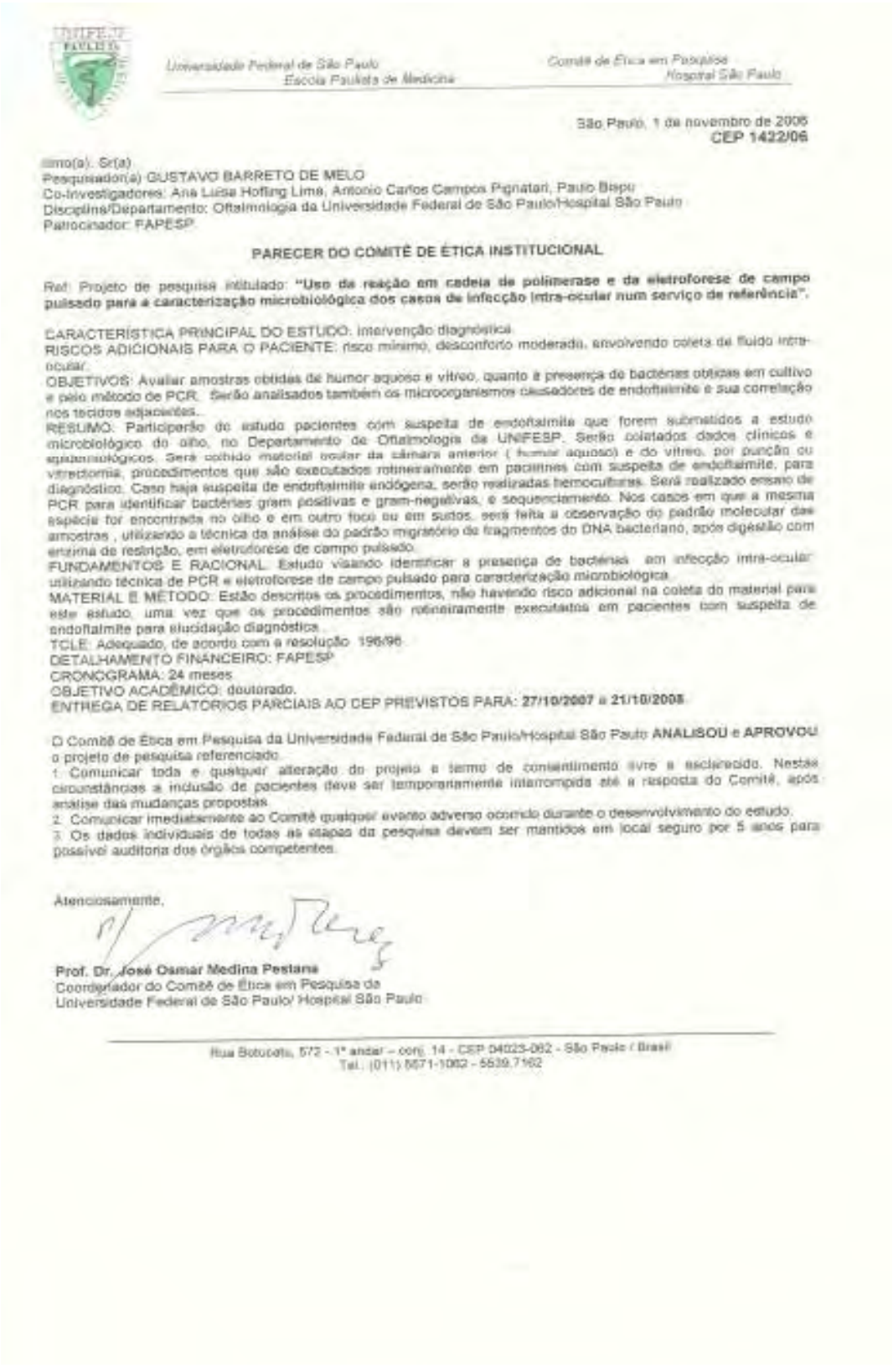
- Como também é uma técnica quantitativa, a reação em cadeia de polimerase em tempo real teve uma nova e distinta aplicação: diferenciação entre contaminação e infecção com base nos valores de limiar do ciclo

Anexo 1 - Parecer do Comitê de Ética em Pesquisa da Unifesp

Anexo 2 - Termo de Consentimento

Anexo 3 - Artigo relacionado publicado como coautor

Anexo 1- Parecer do Comitê de Ética em Pesquisa da Unifesp



Universidade Federal de São Paulo
Escola Paulista de Medicina

Comitê de Ética em Pesquisa
Hospital São Paulo

São Paulo, 1 de novembro de 2006
CEP 1422/06

Ilmo(a). Sr(a)
Pesquisador(a) GUSTAVO BARRETO DE MELO
Co-Investigadores: Ana Luisa Hofling Lima, Antonio Carlos Campos Pignatari, Paulo Bispo
Disciplina/Departamento: Oftalmologia da Universidade Federal de São Paulo/Hospital São Paulo
Patrocinador: FAPESP

PARECER DO COMITÊ DE ÉTICA INSTITUCIONAL

Ref: Projeto de pesquisa intitulado: **"Uso da reação em cadeia de polimerase e da eletroforese de campo pulsado para a caracterização microbiológica dos casos de infecção intra-ocular num serviço de referência".**

CARACTERÍSTICA PRINCIPAL DO ESTUDO: intervenção diagnóstica
RISCOS ADICIONAIS PARA O PACIENTE: risco mínimo, desconforto moderado, envolvendo coleta de fluido intra-ocular.
OBJETIVOS: Avaliar amostras obtidas de humor aquoso e vítreo, quanto à presença de bactérias obtidas em cultivo e pelo método de PCR. Serão analisados também os microorganismos causadores de endoftalmite e sua correlação nos tecidos adjacentes.
RESUMO: Participarão do estudo pacientes com suspeita de endoftalmite que forem submetidos a estudo microbiológico do olho, no Departamento de Oftalmologia da UNIFESP. Serão colatados dados clínicos e epidemiológicos. Será colhido material ocular da câmara anterior (humor aquoso) e do vítreo, por punção ou vitrectomia, procedimentos que são executados rotineiramente em pacientes com suspeita de endoftalmite, para diagnóstico. Caso haja suspeita de endoftalmite endógena, serão realizadas hemoculturas. Será realizado ensaio de PCR para identificar bactérias gram positivas e gram-negativas, e sequenciamento. Nos casos em que a mesma espécie for encontrada no olho e em outro foco ou em surtos, será feita a observação do padrão molecular das amostras , utilizando a técnica da análise do padrão migratório de fragmentos do DNA bacteriano, após digestão com enzima de restrição, em eletroforese de campo pulsado.
FUNDAMENTOS E RACIONAL: Estudo visando identificar a presença de bactérias em infecção intra-ocular utilizando técnica de PCR e eletroforese de campo pulsado para caracterização microbiológica.
MATERIAL E MÉTODO: Estão descritos os procedimentos, não havendo risco adicional na coleta do material para este estudo, uma vez que os procedimentos são rotineiramente executados em pacientes com suspeita de endoftalmite para elucidação diagnóstica.
TCLE: Adequado, de acordo com a resolução 196/96
DETALHAMENTO FINANCEIRO: FAPESP
CRONOGRAMA: 24 meses
OBJETIVO ACADÊMICO: doutorado.
ENTREGA DE RELATÓRIOS PARCIAIS AO CEP PREVISTOS PARA: **27/10/2007** e **21/10/2008**

O Comitê de Ética em Pesquisa da Universidade Federal de São Paulo/Hospital São Paulo **ANALISOU** e **APROVOU** o projeto de pesquisa referenciado.
1. Comunicar toda e qualquer alteração do projeto e termo de consentimento livre e esclarecido. Nestas circunstâncias a indusão de pacientes deve ser temporariamente interrompida até a resposta do Comitê, após análise das mudanças propostas.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do estudo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.

Atenciosamente,

Prof. Dr. José Osmar Medina Pestana
Coordenador do Comitê de Ética em Pesquisa da
Universidade Federal de São Paulo/ Hospital São Paulo

Rua Botucatu, 572 - 1º andar – conj. 14 - CEP 04023-062 - São Paulo / Brasil
Tel.: (011) 5571-1062 - 5539.7162

Anexo 2 - Termo de Consentimento

1 – **Uso da reação em cadeia de polimerase e da eletroforese de campo pulsado para a caracterização microbiológica dos casos de infecção intraocular em um serviço de referência**

2 – O objetivo deste estudo é usar a reação em cadeia de polimerase para a identificação mais precisa da infecção intraocular;

3 – Assim como em todos os casos suspeitos de infecção intraocular, será realizada injeção intraocular para retirada de amostras para identificação de micro-organismos causadores da doença, além da aplicação de antibióticos dentro olho;

4 – A injeção previamente descrita será feita sob anestesia local, com uso de colírios. O paciente poderá sentir leve incômodo durante sua realização. Assim como todo procedimento de injeção no olho, existe risco, apesar de pequeno, de descolamento de retina;

5 – Somente no final do estudo poderemos concluir a presença de algum benefício em acrescentar a reação em cadeia de polimerase na investigação das infecções intraoculares;

6 – Todos os procedimentos realizados para a investigação de infecção intraocular envolvem injeção no olho. Portanto, este estudo não aumenta os riscos além dos já existentes nesses casos;

7 – Garantia de acesso: em qualquer etapa do estudo, você terá acesso aos profissionais responsáveis pela pesquisa para esclarecimento de eventuais dúvidas. O principal investigador é o Dr ***Gustavo Barreto de Melo*** que pode ser encontrado no endereço ***Rua Botucatu, 822*** Telefone(s) 5085-2000. Se você tiver alguma consideração ou dúvida sobre a ética da pesquisa, entre em contato com o Comitê de Ética em Pesquisa (CEP) – Rua Botucatu, 572 – 1º andar – cj 14, 5571-1062, FAX: 5539-7162 – E-mail: cepunifesp@epm.br

8 – É garantida a liberdade da retirada de consentimento a qualquer momento e deixar de participar do estudo, sem qualquer prejuízo à continuidade de seu tratamento na Instituição;

9 – Direito de confidencialidade – As informações obtidas serão analisadas em conjunto com outros pacientes, não sendo divulgado a identificação de nenhum paciente;

10 – Direito de ser mantido atualizado sobre os resultados parciais das pesquisas, quando em estudos abertos, ou de resultados que sejam do conhecimento dos pesquisadores;

11 – Despesas e compensações: não há despesas pessoais para o participante em qualquer fase do estudo, incluindo exames e consultas. Também não há compensação financeira relacionada à sua participação. Se existir qualquer despesa adicional, ela será absorvida pelo orçamento da pesquisa.

12 – Em caso de dano pessoal, diretamente causado pelos procedimentos ou tratamentos

propostos neste estudo (nexo causal comprovado), o participante tem direito a tratamento médico na Instituição, bem como às indenizações legalmente estabelecidas.
13 - Compromisso do pesquisador de utilizar os dados e o material coletado somente para esta pesquisa.

Acredito ter sido suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo "**Uso da reação em cadeia de polimerase e da eletroforese de campo pulsado para a caracterização microbiológica dos casos de infecção intraocular em um serviço de referência**."
Eu discuti com o Dr. ***Gustavo Barreto de Melo*** sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido, ou no meu atendimento neste Serviço.

Assinatura do paciente/representante legal Data __/__/__

Assinatura da testemunha Data __/__/__

para casos de pacientes menores de 18 anos, analfabetos, semi-analfabetos ou portadores de deficiência auditiva ou visual.*(Somente para o responsável do projeto)*

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste paciente ou representante legal para a participação neste estudo.

Assinatura do responsável pelo estudo Data __/__/__

Anexo 3 - Artigo relacionado publicado como coautor

Bispo PJ, Melo GB, Höfling-Lima AL, Pignatari AC. Detection and Gram Discrimination of Bacterial Pathogens from Aqueous and Vitreous Humor Using Real-Time PCR Assays. Invest Ophthalmol Vis Sci. 2010 Aug 11. [Epub ahead of print]

Abstract

Purpose. To develop and apply real-time PCR protocols to detect and classify the Gram status of bacterial pathogens in aqueous and vitreous humor collected from clinically suspected intraocular infections. Methods. The analytical specificity of two PCR assays, SYBR Green 16S rDNA-Based Universal PCR (SGRU-PCR) and a Multiplex Gram-Specific TaqMan-Based PCR (MGST-PCR), was determined using 31 clinically important pathogens, including 20 Gram-positive and 11 Gram-negative. Analytical sensitivity was determined using a 10-fold dilution of *S. epidermidis* and E. coli DNA. Assays were further tested on aqueous (n=10) and vitreous humor (n=11) samples collected from patients with a clinical diagnosis of intraocular infections. Results. DNA was amplified from all control bacterial isolates using SGRUPCR. MGST-PCR correctly classified the Gram status of all these isolates. The limit of detection of *S. epidermidis* and E. coli DNA was 100 fg/microl using SGRUPCR (E = 0.82 and 0.86; r2 = 0.99) and 1 pg/microl for MGST-PCR (E = 0.66 and 0.77; r2 = 0.99. For clinical intraocular samples, positivity of culture was 47.6% and for real-time PCR assays 95.2%. Gram classification was achieved in 100% of PCR-positive samples using MGST-PCR. Among microbiologically negative samples, real- time PCR assays were positive in 90% of cases. False positive rate in control aqueous was 3.2% and control samples of vitreous were negative. Conclusions. Real-time PCR assays demonstrated a good correlation with culture-proven results. With the use of these methods, bacterial detection was improved from 47.6% to 95.3%, demonstrating to be sensible and fast tests for bacterial endophthalmitis diagnosis.

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ABSTRACT

Purpose: I- To report the incidence of bacterial endophthalmitis and the frequency of its causative microorganisms at a university-setting in Brazil. **II-** To assess the distribution of microorganisms isolated from patients with bacterial endophthalmitis and their antimicrobial susceptibility. **III-** To determine the usefulness of real-time polymerase chain reaction (PCR) assays in the diagnosis of post-operative bacterial endophthalmitis in clinically diagnosed infectious cases and to test for bacterial DNA in control samples.

Methods: I- Main data assessed from cases of presumed postoperative bacterial endophthalmitis from 2002 to 2008 were: number of cataract surgeries performed, incidence of endophthalmitis, positivity of Gram staining and culture (aqueous and/or vitreous), and antimicrobial susceptibility. **II-** Retrospective analysis of medical and microbiological records of patients with suspected diagnosis of endophthalmitis was carried out. The following information was assessed: number of clinically diagnosed and culture-positive endophthalmitis cases, predisposing factors for the infection, Gram staining and culture outcomes (aqueous and/or vitreous), microbial characterization and frequency, and antimicrobial susceptibility. **III-** Samples from eyes of patients with clinically diagnosed infectious endophthalmitis after cataract surgery were included, as well as vitreous and aqueous samples from eyes without infection or inflammatory reaction. Universal and Gram-specific real-time PCR were carried out and the technique's sensitivity and cycle thresholds were determined. Gram staining and culture were also assessed.

Results: I- Seventy-three eyes of 73 patients (43 females and 30 males) developed presumed infectious endophthalmitis after 24,590 cataract surgeries. The incidence decreased from 0.49% in 2003 to 0.17% in 2006 and stabilized afterwards. Coagulase negative *Staphylococci* (CoNS) and *Streptococcus viridans* (56.5% and 15%, respectively) were the most common bacteria. Culture and Gram stain were negative in 36.9%. CoNS presented susceptibility rates of 80%-sensitivity to oxacillin, 90% to fourth-generation quinolones and 100% to vancomycin. **II-** A hundred and seven (46%) of 231 patients with bacterial endophthalmitis showed positive results by gram-stain or culture. Of these, 97 (42%) patients were positive for culture only. Most of them (62%) were secondary to a surgical procedure (postoperative), 12% were post-traumatic and 26% were secondary to an unknown source. A total of 100 microorganisms were isolated (38 aqueous and 67 vitreous samples) from the 97 culture-positive cases. Coagulase-negative *Staphylococcus* (CoNS) (48%) were most frequently isolated, followed by *Streptococcus viridans* (18%) and *Staphylococcus aureus* (13%). Antimicrobial susceptibility for CoNS was as follows: amikacin - 91.6%, cephalothin - 97.9%, ceftriaxone - 50%, ciprofloxacin - 62.5%, chloramphenicol - 91.8%, gatifloxacin - 79.5%, gentamicin - 72.9%, moxifloxacin - 89.5%, ofloxacin - 70.8%, oxacillin - 58.3%, penicillin - 33.3%, tobramycin - 85.4%, and vancomycin - 100%. **III-** Eleven patients with infectious endophthalmitis (9 vitreous and 7 aqueous samples) after cataract surgery were included, as well as 12 vitreous and 50 aqueous samples from control eyes. It was possible to identify 80% and 75% of the patients with infectious endophthalmitis by Gram staining and culture, respectively. Real-time PCR assays were positive in 91% of the patients, using either aqueous and/or vitreous samples. None of the 12 vitreous controls were positive and two of the aqueous control samples were positive by real-time PCR. The Ct cutoff value for universal-PCR was 36 (sensitivity: 93.8%; specificity: 100%) and 38 for Gram-specific-PCR (sensitivity: 93.8%; specificity: 100%). Gram-positive microorganisms prevailed and visual acuity varied according to the causative bacteria. **Conclusions: I-** The rate of bacterial endophthalmitis, microbial isolates and antibiotic susceptibility are in accordance with

the literature. Despite using prophylactic antibiotic drops, organisms from infected cases susceptible to the antibiotics topically applied were found. **II-** Gram-positive bacteria were the major causes of infectious endophthalmitis. CoNS was the most common isolate. Susceptibility to oxacillin and fourth-generation quinolones was lower than previously published. **III-** Real-time PCR is a fast and sensitive diagnostic tool in cases of bacterial endophthalmitis. As a quantitative technique, it may also serve as a new and unique application: the distinction between contamination and infection based on the cycle threshold value.

Keywords: endophthalmitis; epidemiology; diagnosis; polymerase chain reaction; microbial sensitivity tests.