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Identification of Torque Teno Virus in Culture-Negative Endophthalmitis by Representational Deep DNA Sequencing

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Purpose: To test the hypothesis that uncultured organisms may be present in cases of culture-negative endophthalmitis by use of deep DNA sequencing of vitreous biopsies.

Design: Single-center, consecutive, prospective, observational study.

Participants: Aqueous or vitreous biopsies from 21 consecutive patients presenting with presumed infectious endophthalmitis and 7 vitreous samples from patients undergoing surgery for noninfectious retinal disorders.

Methods: Traditional bacterial and fungal culture, 16S quantitative polymerase chain reaction (qPCR), and a representational deep-sequencing method (biome representational in silico karyotyping [BRiSK]) were applied in parallel to samples to identify DNA sequences corresponding to potential pathogens.

Main Outcome Measures: Presence of potential pathogen DNA in ocular samples.

Results: Zero of 7 control eyes undergoing routine vitreous surgery yielded positive results for bacteria or virus by culture or 16S polymerase chain reaction (PCR). A total of 14 of the 21 samples (66.7%) from eyes harboring suspected infectious endophthalmitis were culture-positive, the most common being *Staphylococcal* and *Streptococcal* species. There was good agreement among culture, 16S bacterial PCR, and BRiSK methodologies for culture-positive cases (Fleiss' kappa of 0.621). 16S PCR did not yield a recognizable pathogen sequence in any culture-negative sample, whereas BRiSK suggested the presence of *Streptococcus* in 1 culture-negative sample. With the use of BRiSK, 57.1% of culture-positive and 100% of culture-negative samples demonstrated the presence of torque teno virus (TTV) sequences, compared with none in the controls ($P = 0.0005$, Fisher exact test). The presence of TTV viral DNA was confirmed in 7 cases by qPCR. No other known viruses or potential pathogens were identified in these samples.

Conclusions: Culture, 16S qPCR, and BRiSK provide complementary information in presumed infectious endophthalmitis. The majority of culture-negative endophthalmitis samples did not contain significant levels of bacterial DNA. "Culture negativity" does not seem to be due to failure of growth of fastidious bacteria. The small DNA virus TTV was unexpectedly found in all culture-negative samples and some culture-positive samples. This study cannot distinguish whether TTV is a direct intraocular pathogen, an adjuvant for inflammation, a general marker of inflammation, or a commensal virus but provides a testable hypothesis for a pathogenic mechanism in culture-negative endophthalmitis. *Ophthalmology 2015;122:524-530 © 2015 by the American Academy of Ophthalmology.*

Infectious endophthalmitis is among the most serious postsurgical complications of ophthalmic surgery. Although a rare complication of cataract surgery or intravitreal injection, with an incidence of 0.05% to 0.3%,[1–7] endophthalmitis often leads to poor visual outcomes.[3,8,9] Because of the high volume of current and anticipated cataract surgery worldwide (with VISION2020 goals of 32 million cataract surgeries per year), and the large and increasing number of intravitreal injections performed, endophthalmitis will continue to affect tens of thousands of individuals annually worldwide.

The standard technique for diagnosing endophthalmitis is microbial culture. Despite the unambiguous presentation of most cases of postoperative endophthalmitis, microbial culture has a yield of approximately only 70%.[9] More recent studies examining endophthalmitis after intravitreal injection have found less than 50% of cases to be culture-positive.[10,11] In recent years, several studies have examined the utility of bacterial ribosomal 16S polymerase chain reaction (PCR) and sequencing in identifying bacterial pathogens in endophthalmitis.[12–18] In this technique, a set of DNA primers that recognize the conserved 16S ribosomal gene found in nearly all bacteria are used to detect the presence of bacterial DNA. Polymerase chain reaction products then can be sequenced to determine the genus of bacteria present. These studies have shown that 16S PCR is more sensitive and specific than traditional culture techniques. However, 16S PCR has significant limitations: Its sensitivity is

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sufficiently high that false-positive and artifactual products may be produced,[19] and determination of the causative organism requires subsequent sequencing or further analysis of PCR products. These limitations can be overcome by using quantitation via quantitative PCR (qPCR) combined with sequencing of product; however, this approach rarely has been used to date in the study of endophthalmitis.[14] In addition, 16S amplification is limited to bacteria and cannot detect fungi (which require separate PCR of fungal ribosomal DNA sequences), parasites, or viruses.

With the advent of massively parallel DNA sequencing platforms, the availability of the complete sequence of the human genome, and increasing computational capacities, it is becoming possible to sequence all DNA in a biopsy sample and identify all nonhuman DNA present to detect potential occult or novel pathogens. At present, it remains prohibitively labor- and cost-intensive to completely sequence all genomes present in routine biopsy samples. However, it is possible to purify a defined fraction of all DNA present in a sample and sequence this to near saturation. One technique for achieving this is biome representational in silico karyotyping (BRiSK).[20] This technique is capable of identifying most known bacteria, as well as phage, viruses, and previously unknown organisms. We report the application of deep DNA sequencing to vitreous and aqueous biopsies from patients with endophthalmitis and compare the results from this technique with traditional culture and 16S qPCR. We find that BRiSK has sensitivity and specificity comparable to the culture and qPCR techniques. We unexpectedly have identified DNA from a known anellovirus (torque teno virus [TTV]) in a majority of endophthalmitis cases, including all culture-negative endophthalmitis cases.

Methods

This prospective study was approved by the Wills Eye Hospital Institutional Review Board and the University of Washington Human Studies Division Institutional Review Board. Research adhered to the tenets of the Declaration of Helsinki and was conducted in accordance with Health Insurance Portability and Accountability Act regulations. Written informed consent was obtained from all subjects for testing of samples. All participants were enrolled from the clinical offices of the Retina Service of the Wills Eye Hospital. In a prospective fashion, 21 consecutive patients diagnosed with infectious endophthalmitis on the basis of clinical history and examination by a retina specialist (S.J.G.) at Wills Eye Hospital were enrolled. Sample size for this pilot study was chosen to provide at least 5 culture-negative samples for analysis and limited by the cost of deep DNA sequencing (at ~$1000/sequencing run). The clinical diagnosis of endophthalmitis was made on the basis of a combination of ocular pain, conjunctival injection, anterior chamber cellular reaction, and posterior vitritis. Vitreous tap or aqueous tap was obtained in standard clinical fashion after povidone-iodine antisepsis. As a control group, 7 consecutive patients with uninflamed eyes undergoing vitrectomy for noninfectious retinal disorders (epiretinal membrane or macular hole) provided consent for vitreous tap done during the procedure. Briefly, the vitreous samples in the control group were obtained as follows: Patients with epiretinal membranes or full-thickness macular holes were included. After informed consent was obtained, patients were prepped and draped in the usual sterile fashion for intraocular surgery. A 23-gauge vitrectomy system (Constellation; Alcon, Fort Worth, TX) was used in all cases. The cannulas were placed 3.5 to 4 mm posterior to the limbus in a beveled fashion. The infusion line was affixed to the inferotemporal quadrant but not turned on. The vitrectomy instrument was introduced, and using proportional vitrectomy at 5000 CPM with manual aspiration, approximately 0.25 ml of vitreous was removed and included in the study. In all cases, approximately 0.2 to 0.3 ml of vitreous humor sample was sent for standard microbiological culture (including blood agar, chocolate agar, Sabouraud's medium, and thioglycolate broth), and the remaining 0.05 to 0.10 ml was immediately frozen for subsequent analysis by 16S PCR and BRiSK.

DNA Purification

Genomic DNA was isolated from 20 to 50 μl of vitreous humor or aqueous fluid using the DNeasy Blood & Tissue Kit (Qiagen, Inc., Venlo, the Netherlands) as per protocol. The DNA was eluted in the kit elution buffer and stored at −20°C. DNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Phi29 of the genomic DNA was amplified using the REPLI-g Mini Kit (Qiagen, Inc.) as per instructions. The Phi-amplified genomic DNA was also stored at −20°C.

16S Bacterial, Torque Teno Virus, and Actin Quantitative Polymerase Chain Reaction

Pan bacterial PCR was performed using 16S ribosomal RNA universal primers (Integrated DNA Technologies, San Diego, CA). The sequences of the primers were 5′-GAGGAAGGTGGGG ATGACGT-3′ and 5′-AGGCCCGGGAACGTATTCAC-3′. HotStarTaq plus DNA polymerase was used for the PCR reactions. For each reaction, 100 ng of genomic DNA was used. The master mix, containing 10× buffer, Taq polymerase, deoxynucleotide triphosphate mix, and the primers, was treated with 8-methoxypsoralen (25 μg/ml) and ultraviolet nicked for 5 minutes (Bio-Rad GS gene linker, ultraviolet chamber; Bio-Rad Laboratories Inc., Hercules, CA) to bind any contaminating DNA. Polymerase chain reaction was amplified in a MasterCycler gradient (Eppendorf, Hamburg, Germany). Cycling conditions were 10-minute denaturation at 94°C, followed by 30 cycles of 45-second denaturation at 94°C, 30-second annealing at 58°C, and 1-minute extension at 72°C. The elongation step was 10 minutes at 72°C.

The primer pairs for TTV PCR were 5′-AGGTGAGT TTACACACCGCAGTCA-3′ and 5′-AATGAAGACCCTAAGAG CCTTGCC-3′. The primers for β-actin were 5′-TGCTCCTCC TGAGCGCAAG-3′ and 5′-GCCGGACTCGTCATACTCC-3′. Cycling conditions for the TTV primers were 10-minute denaturation at 95°C, followed by 25 cycles of 30-second denaturation at 95°C, 15-second annealing at 58°C, and 1-minute extension at 72°C. Final elongation was 10 minutes at 72°C.

Quantitative PCR assay was performed on the Applied Biosystems (Foster City, CA) 7500 Fast Real-Time PCR system. The final PCR mix contained 0.8 μl each of forward and reverse primers (final concentration of each, 0.4 mmol/l), 10 μl of the Absolute Blue qPCR SYBR low ROX Mix (Thermo Fisher Scientific, Waltham, MA), and 1 μl of unamplified genomic DNA. For 16S DNA PCR, the master mix without the template was treated with 8-methoxypsoralen and ultraviolet treated for 5 minutes. The final reaction volume was 20 μl. For standard curve, a plasmid complementary DNA of the cloned gene of interest (e.g., the target sequence for 16S, TTV, or actin) was serially diluted 10-fold to obtain copy numbers ranging from 1×10^1 to 10^8 copy/ml. Quantitative PCR routinely was able to detect 10 copies/ml of each control complementary DNA. The run consisted of initial holding