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Emerging techniques for pathogen discovery in endophthalmitis

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Abstract

Purpose of review—Despite the inability to detect certain organisms and relatively low yield, microbial culture is the current gold standard for the diagnosis of most intraocular infections. Research on alternative molecular diagnostic methods has produced an array of strategies that augment and improve pathogen detection. This review summarizes the most recent literature on this topic.

Recent findings—The yield of traditional microbial culture has not improved since the Endophthalmitis Vitrectomy Study results were published 20 years ago. Advances in polymerase chain reaction (PCR) methods have enabled quantification of pathogen load and screening for multiple organisms at once. More recently, deep sequencing techniques allow highly sensitive detection of any DNA-based life form in a specimen. This offers the promise of not only improved detection of traditional organisms but can also identify organisms not previously associated with endophthalmitis.

Summary—Molecular diagnostic methods enhance the results of microbial culture and may become the new standard in the diagnosis of intraocular infections.

Keywords

endophthalmitis; polymerase chain reaction; PCR; Biome Representational in Silico Karyotyping; BRiSK; ocular surface microbiome

INTRODUCTION

Infectious endophthalmitis is a vision-threatening and potentially devastating intraocular inflammatory process that can occur after intraocular surgery, trauma, and from non-ocular endogenous infections. The ability to identify the causative pathogen(s) has wide-reaching implications for treatment, as rapid and accurate identification of the organism guides further anti-microbial selection and potentially surgical management. A body of research has described a number of new, molecularly-based diagnostic techniques, which offer several

advantages over traditional methods of microbial detection. We review the salient literature published on techniques for pathogen discovery in endophthalmitis.

THE CURRENT GOLD STANDARD: STAIN AND CULTURE

The management of patients with post-cataract endophthalmitis has changed little since the publication of the Endophthalmitis Vitrectomy Study (EVS) in 1995 [1]. The EVS showed that nearly 30% of vitreous culture specimens were culture-negative despite exhaustive culturing attempts. Specimens were plated on chocolate agar, fresh Sabouraud dextrose agar, and in freshly reduced, enriched thioglycolate liquid. Gram stains were prepared both from anterior chamber and undiluted vitreous specimens. The filtered vitreous effluent (collected in the vitrectomy cassette) was divided into three pieces and similarly cultured. Finally, anaerobic culture was performed in either enriched thioglycolate broth or anaerobic blood agar enriched with hemin and vitamin K at 37°C.

The methods used in the EVS are a fairly standard protocol for the culture of intraocular specimens though variations exist. Interestingly, although the EVS study was able to detect microbes in approximately 70% of cases, subsequent studies have had more variable success, with culture-positive rates ranging from 34%–71% after clear corneal cataract surgery [2–5] and 60% after pars plana vitrectomy [6]. Culture positive rates from post-trauma endophthalmitis have wide variability ranging from 38%–64% [7, 8], as well as after intravitreal anti-VEGF injection (30–39%) [9–11]. The published data on positive Gram stain also is poor with rates of microbial isolation ranging from 29% to 36% [12,13], and most recently as low as 7.5% [14]. To muddy the picture further, the vast majority of studies examining endophthalmitis only characterize the isolates that are recovered and do not report what percentage of patients have positive cultures. As such, it is not possible to accurately estimate sensitivity and specificity of culture techniques. It is difficult to determine whether these wide-ranging success are due to study design, population, or variations in laboratory protocol or materials but they nonetheless demonstrate room for improvement.

Despite their limitations, traditional stain and culture techniques are widely available and relatively inexpensive, and have a long history of use. Interpretation is generally straightforward. Gram stain results are immediately available when positive, but more often than not definitive diagnosis is delayed because cultures can be slow to grow, or may not grow at all. As a result, many cases of suspected intraocular infections are treated based on characteristic appearance (e.g., ocular toxoplasmosis), indirect evidence of an organism (e.g., positive syphilis serum titers for syphilis), or clinical appearance (e.g., post-cataract endophthalmitis).

POLYMERASE CHAIN REACTION (PCR)

Although previously used for research, PCR is used to help diagnose cases of anterior uveitis [15, 16], keratitis [17, 18], and viral retinitis [19, 20], and has shown promise for the management of endophthalmitis. PCR is based on the amplification and subsequent detection of deoxyribonucleic acid (DNA) in a biological sample through the use of a thermostable DNA polymerase that rapidly replicate short sequences of DNA [21–23]. Its

first use in ophthalmology was described in 1993 for cytomegalovirus retinitis [24] and it plays an important role for detection of intraocular viral infection.

Briefly, amplification of DNA is based on 30–40 cycles of three steps: (1) denaturation, whereby a brief application of heat is employed to separate the two DNA strands of an organism into two single strands; (2) primer hybridization, whereby excessive amounts of pre-selected primers are added to hybridize to their complementary sequences on the single-stranded DNA; and (3) elongation, whereby a DNA polymerase synthesizes a complementary strand of DNA adjacent to the hybridized primers. A thermocycler automatically performs these steps and with each cycle the amount of DNA increases exponentially. A single starting copy will theoretically produce over 30 billion copies after 35 cycles. A single, 200 base pair segment of DNA weighs only about 2×10^{19} grams (or 20 attograms); however after 35 cycles of amplification, 7.5 nanograms theoretically will be produced, a quantity which can be analyzed by electrophoresis or DNA sequencing. Thus PCR theoretically has sensitivity to detect single molecules.

Detection of the amplified DNA has conventionally been via agarose gel electrophoresis paired with an intercalating agent (e.g. ethidium bromide) that fluoresces under ultraviolet illumination. However, these conventional techniques only provide qualitative data and detect the presence or absence of a DNA-segment of appropriate length. As such, post-PCR analysis, the most common of which are DNA sequencing (via comparison against established databases), in situ hybridization, and an enzymatic method using restriction fragment length polymorphisms to identify corresponding microbial species become necessary.

All bacteria have conserved 16S ribosomal DNA sequences that are unique to the kingdoms of archaea and bacteria. By identifying 16S ribosomal DNA in a specimen, PCR can detect the presence of bacteria with sensitivities ranging 66–95%, compared to 34–53% for culture [25,26,2]. Fungi also have a common ribosomal 18S/28S DNA sequence that enables detection of this class of organisms via PCR. Subsequent sequencing of resultant PCR fragments can determine the precise pathogen.

In ‘standard’ clinical PCR, each organism or class of organisms under consideration must be ordered individually and requires its own portion of sample (typically 10 to 50 ul). For some differential diagnoses, there may be insufficient material to test for all suspected pathogens. There is no commercially available kit that looks for the full breadth of most pathogens associated with endophthalmitis, which currently means that the clinician’s degree of suspicion and clinical acumen influence the pretest and posttest probabilities of certain studies [27]. Thus the advanced methods described above depend on a priori knowledge or suspicion of a particular species to choose appropriate primers, tags, and targets for analysis.

TECHNICAL ADVANCES

Improvements in DNA extraction and purification have facilitated the recovery of DNA that is free of the molecules and proteins that normally decrease the yield of PCR techniques. This has paved the way to use PCR on very small-volume samples [28], which will make the lab result of “quantity not sufficient” increasingly rare.

Multiplex PCR, or PCR using novel primer sets for a panel of common pathogens, has enabled simultaneous performance of PCR for cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), and *Toxoplasma gondii*, with minimal loss of sensitivity and with high specificity [29]. This makes it possible to concomitantly run one high-quality PCR reaction for the detection of multiple organisms in the place of several separate reactions, saving both time and resources and expediting the results of these tests.

Quantitative PCR (qPCR) simultaneously amplifies DNA and tags each DNA copy with a fluorescent molecule, allowing the amount of pathogen in a sample to be quickly measured by the level of fluorescence detected [30]. Other qPCR methods may use a third primer (or 'TaqMan' probe) to quench fluorescence. In both cases, by using calibration curves a precise estimate of the amount of starting DNA from the organism in question can be determined, which helps distinguish DNA from a bacterial contaminant from true infection.

These technical advances have begun to make their way into clinical care. One recent study [31] of 433 PCR tests of 143 aqueous and vitreous humor specimens showed that PCR results had a positive predictive value of 98.7% in those with suspected infectious chorioretinitis, which altered the therapeutic approach in 25% of cases and led to clinical resolution after therapy was changed.

Both qPCR and variations of multiplex PCR will likely find their way into widespread use and may soon replace several different PCR tests with a few panel tests.

Researchers in Japan have recently led the way in developing novel PCR methods for detection of fungal organisms in endophthalmitis, using broad-range, real-time PCR for fungal infections [32, 33]. "Broad range" refers to the primers that have broad interspecies specificity (e.g. 28S ribosomal DNA in fungi), and "real time" is synonymous with "quantitative" PCR in its ability to amplify and detect amplification simultaneously.

Until recently, no one had succeeded in creating a single, comprehensive protocol for the PCR detection of most known pathogenic microbes. Sugita et al recently reached this goal by using a combination of broad-range bacterial and fungal PCR and multiplex PCR to simultaneously assay for herpes viruses, bacteria, fungi, and toxoplasma in 500 inflamed eyes. Though the assay was not perfect, it had a positive predictive value of 99% and a negative predictive value of 93%, much higher than is possible with traditional culture. [34] This technique is potentially powerful, but it does not yet yield real-time results. Although PCR amplification using this combination approach can be done relatively quickly (several hours), ascertaining the species of a particular microbe using current post-PCR analyses could take up to several days in most centers.

With the advent of more refined comprehensive PCR protocols and possible coupling with qPCR, rapid identification of pathogenic microbes in endophthalmitis is on the horizon.

MASSIVE PARALLEL SEQUENCING

Recently, the Van Gelder laboratory developed a representational deep sequencing technique called Biome Representational in Silico Karyotyping (BRiSK) that allows

sensitive detection of any DNA-based life form.[35] In this technique, DNA is purified from a biopsy sample, and then digested with a specific restriction endonuclease (BsaXI) that releases a 33 base pair fragment from approximately every 4,000 base pairs of the starting DNA. This 0.8% of the DNA (i.e. 33/4,000) is then sequenced using a massively parallel DNA sequencing apparatus, as was used to sequence the human genome. The short 33 bp DNA 'tags' that result can readily be assigned to human DNA, known pathogen DNA, or novel DNA (i.e., new pathogen). The sequenced tags are then compared against a large database generated from the NCBI Genbank containing *all known* DNA sequences. The database is updated daily to incorporate the most current available data and is able to identify tags from mammalian, bacterial, fungal, parasitic, and viral organisms.

There are several advantages to this technique. First, because BRiSK sequences a representation of all DNA present within a sample, it allows a comprehensive evaluation of all organisms and can potentially lead to discovery of pathogens that have not been associated with disease states. This is unlike PCR where a particular organism must be known and suspected in order to be detected. Second, it can be performed with the small sample sizes obtained in ophthalmology. With phi29 amplification, one nanogram of DNA sample (typically less than 0.1mL) is sufficient for BRiSK. Third, next-generation sequencing enables quantification of the organism(s), and the relative abundance of each organism in the specimen can be determined. Fourth, the technique is relatively quick. BRiSK technology takes approximately 24 hours after the preparation of DNA samples.

This has the potential to make the clinician's life much better. To illustrate the role that BRiSK could place, a physician might examine a patient with a presumed infectious corneal ulcer or a case of endophthalmitis but the clinical examination or history might not suggest a particular organism that can be easily cultured or tested with PCR. In addition, the clinician may want to test for a pathogen with a small sample. Using BRiSK, the sample is compared against the database of all organisms that have been ever sequenced. In contrast to traditional PCR in which the physician gets a report listing the test results of the few organisms that the doctor was able to screen for, with BRiSK the clinician receives a list of all organisms present and their respective abundance in the presumed infectious sample.

A few studies have used BRiSK to detect organisms *de novo* [36, 37]. We performed a pilot study with 21 consecutive vitreous samples from post-procedural, presumed infectious endophthalmitis and 7 vitreous samples from routine vitrectomy cases without inflammation were compared with culture, 16S PCR, and BRiSK [36]. Fourteen of the 21 endophthalmitis samples were found to be bacterial culture-positive. Culture, 16S PCR results, and BRiSK identified identical or closely related species in all culture-positive cases and there was good agreement in bacterial identification between all 3 tests (kappa=0.62). Seven of the 21 endophthalmitis samples were found to be culture-negative and 16S PCR also failed to demonstrate significant levels of bacterial DNA in any of the culture-negative cases, essentially confirming the culture results. Unexpectedly, all 7 of the culture-negative endophthalmitis samples had substantial amount of DNA tags for the human anellovirus also called the Torque teno virus (TTV) suggesting that this unexpected virus is potentially pathogenic, immunogenic, or a marker of inflammation. In addition, numerous tags that did not match any known sequences in NCBI Genbank database. These unknown sequences

may represent novel pathogens that have not been identified before. As we move forward with this technology, new pathogens may be found leading, to better understanding of previously defined “sterile endophthalmitis.”

BRiSK is ideal in analyzing the array of microbes in a sample without *a priori* knowledge. Thus, it has been used in characterizing different microbiomes on a species level. Examples of this application include determining the gut microbiome in HLA-B27 transgenic rats [37] and conjunctival ocular surface microbiome studies. After reviewing the results of BRiSK, the organisms of interest can be determined, then other sequencing methods such as 16S PCR, real time PCR, or whole genome sequencing can be carried out as adjunctive techniques.

There are several limitations of BRiSK. First, BRiSK cannot detect any non-DNA based life forms such as RNA viruses, and its sensitivity is limited to the identified tags (DNA sequences) within the database. Second, the strength of the detection of organisms depends largely on the relative abundance and the genomic size of the organisms. As with any sequencing technique, careful attention must be given to decrease contamination and false positivity, particularly when studying comparatively few organisms in any particular microbiome. On the other hand, BRiSK may overestimate the number of organisms if they have a long sequence with multiple BSaXI recognition sites. The technique is currently relatively expensive (several hundred dollars per sample) and requires use of a massively parallel DNA sequencing methodology. However, BRiSK is not as costly and time consuming as whole genome sequencing; and costs are likely to continue to fall with advances in sequencing technology.

CONCLUSION

With the advances in molecular diagnostic methods and sequencing technology, the analysis of endophthalmitis is being rapidly expanded. We are no longer bound by traditional stain, culture, and uniplex PCR, especially as further technical advances make the next generation of PCR and massive parallel sequencing more common.

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Key Points

In endophthalmitis, the ability to identify the causative pathogen(s) has wide-reaching implications for treatment, and exciting research is emerging that has several advantages over traditional methods of microbial detection.

The management of patients with post-cataract endophthalmitis has changed very little since the publication of the Endophthalmitis Vitrectomy Study in 1995.

For cases of post-cataract endophthalmitis, only around 70% of samples demonstrate bacterial growth, and the rates are even lower for post-intravitreal injection endophthalmitis.

Technical advances in polymerase chain reaction are paving the way for more efficient, sensitive, and specific identification of pathological organisms.

Massive parallel sequencing is very useful for analyzing the array of microbes in a sample without *a priori* knowledge, which makes it a powerful tool for characterizing different microbiomes at a species level and enables sensitive detection of any DNA-based life form.