POLYMERASE CHAIN REACTION (PCR) AS AN AID IN DIAGNOSIS IN INTRAOCULAR INFLAMMATION

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INTRODUCTION

First described by Saiki et al [1] in 1985, polymerase chain reaction (PCR) is a biochemical technique that allows for the detection of infinitesimal amounts of specific nucleic acids.[2]

Since the introduction of its modern form in 1988,[3] PCR has revolutionized much of molecular biology and has greatly accelerated the development of molecular diagnostics. Kary B. Mullis from USA received a Nobel Prize in 1993 for inventing this technique. PCR has been used to diagnose viral uveitis, infectious endophthalmitis and protozoan eye disease.[4] This is a review of the use of PCR to find the etiology for relevant management in intraocular inflammation.

BIOCHEMICAL BASIS OF PCR

PCR is a technique involving enzymatic amplification of nucleic acid sequences in repeated cycles of denaturation, oligonucleotide annealing and DNA polymerase extension.[5]

To perform PCR, one must have starting material (i.e., an aqueous or vitreous biopsy specimen), a pair of short synthetic oligonucleotide primers complementary to the DNA sequence being amplified, appropriate buffers, nucleotide triphosphates, a thermostable DNA polymerase and a thermal cycling machine. In the thermal cycling machine, the initial temperature is set around 94° C at which denaturation of the DNA strands into 2 single strands takes place. The thermal cycler is then set at the annealing temperature, usually in the range of 55°C. At this temperature the preselected primers flank the specific area of interest on the DNA. The temperature is then raised to around 72-74° C, at which the DNA polymerase gets activated and DNA complementary to the target sequence is synthesized. When the temperature is raised to 94° C the strands dissociate and the cycle begins once again. Typically 30 to 40 cycles of PCR are employed.[2]

With each cycle there is doubling of the final desired DNA product. Thus amplification of 2^N will occur after N thermal cycles. A 35-cycle amplification will theoretically yield 34 billion DNA molecules for each starting molecule.[2, 6]

The products of PCR are detected usually by visualizing the DNA molecules, separated by size, using agarose or

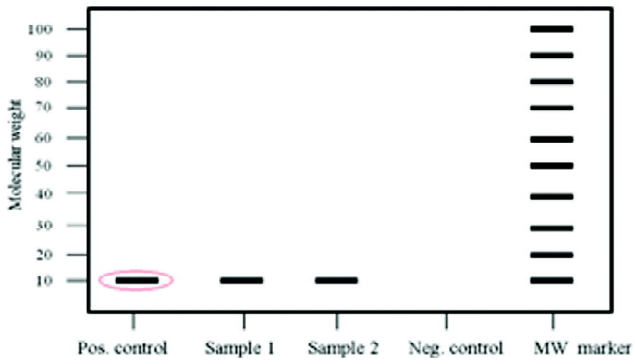


Fig. 1a- Schematic diagram of agarose gel of PCR products.

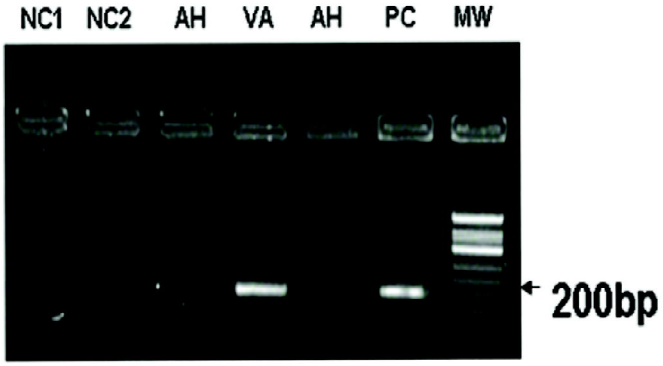


Fig. 1b- Agarose gel electrophoretogram showing amplified products of PCR to detect Mycobacterium tuberculosis genome; NC 1- Negative control 1; NC 2 - Negative control 2; AH - Acqueous humor sample; VA-Vitreous aspirate; PC- Positive control; MW.- Molecular weight; bp- Base pairs.

acrylamide gel electrophoresis. Confirmation of the identity of the PCR product can be achieved by digesting the product with restriction endonuclease and observing the restriction digest pattern, a technique called *fingerprinting*. Ultimate identification of a DNA fragment can be achieved by sequencing the PCR product DNA.

The whole process from sample acquisition to visualizing the products takes only 2-3 hours.[2]

SAMPLE PREPARATION FOR PCR

PCR can be performed on nearly any ocular specimen or biopsy. For the diagnosis of uveitis, the obtained sample is usually an anterior chamber paracentesis, vitreous tap or intraocular fine needle aspiration biopsy (FNAB). Anterior chamber paracentesis of 50 microlitres is usually sufficient for diagnostic purposes. For the vitrectomy specimens, 100-500 microlitres of initial undiluted, pre-infusion aspirate are preferred. Specimens should be aseptically transferred to a new, sterile, plastic microfuge vials (commercially available) and quick-frozen at -20° C or at -80° C. The sample should remain frozen until processed, since freeze thaw cycles will release nucleases that will degrade all RNA and DNA.[7]

STRENGTHS AND PITFALLS OF PCR

The sensitivity for detection of foreign DNA is very high. Thus PCR is potentially more sensitive and also faster than culture for detection of many organisms.[8] PCR is also phenomenally specific test capable of single base pair mismatch specificity.[2] Although PCR would seem to have nearly ideal characteristics for a diagnostic test, the high sensitivity and specificity can cause significant pitfalls.

The very high sensitivity of PCR makes it prone to false-positive results from laboratory contamination. Also PCR can detect dead, latent or colonizing commensal organism.

The high specificity in turn can lead to false-negative results if the organism's target DNA location is polymorphic.

Also PCR cannot detect organism for which primer pairs have not been provided in the reaction. Thus the biggest pitfall of PCR is that a narrow, well-defined differential diagnosis is required for it to be effectively useful.

VARIATIONS OF PCR

-Real-time PCR

It is the real-time version of quantitative PCR (Q-PCR), a modification of PCR. It is used to determine whether or not a specific sequence is present in the sample; and if present, the number of copies in the sample. It is used to differentiate between active infection and latent pathogen by quantifying the number of pathogen genomes in a sample.

-Reverse Transcriptase PCR (RT-PCR)

PCR is an effective tool for amplifying DNA. To measure RNA, the RNA sample first needs to be reverse transcribed to DNA using an enzyme reverse transcriptase. This transcribed DNA is known as cDNA or complementary DNA. This method known as reverse transcriptase PCR requires extensive optimization of PCR cycles. This method can be used to distinguish between active and latent infection by an organism like Toxoplasma gondii and Herpes virus by assaying the tissue for actively transcribed RNA from the pathogen.

-Nested PCR (nPCR)

It involves two sets of primers used in two successive runs of PCR, with the second set intended to amplify a secondary target within the first product. Thus it can be used to reduce contaminations in products due to amplification of unexpected primer binding sites.

-Universal primer PCR

Certain genes are common between different species of the same type of organisms. Thus primers aimed at these common genes can be designed and used for detecting the organism. The 16S ribosomal RNA sequence represents the eubacterial genome and 18S and 28S ribosomal RNA genes represent the panfungal genome since they are present in all bacteria and fungi respectively.[8-11] Primers to these can be used to detect presence of any bacterium or fungus. Final identification of the specific species can then be done by PCR based restriction fragment length polymorphism (RFLP) or array hybridization.

-Multiplex PCR

This is used when there is no universal primer for a group of organisms. In this case multiple primer sets are combined to test for multiple pathogens simultaneously, by amplification of any one of the primer sets. The detection of herpes virus family in a sample can be done by multiplex PCR.

PCR IN INTRAOCULAR INFLAMMATION

Diagnosis of infectious uveitis

PCR has had a major impact on our ability to detect infectious agents. Since the first detection of Toxoplasma gondii DNA in ocular tissue with the use of PCR in 1990, [12] PCR has been applied to the detection and diagnosis of various infectious uveitis.

The initial application of PCR diagnostics to ophthalmic disease was in the detection of viral uveitis. [7, 13-16]Over time it has been shown that PCR is superior in viral infections as compared to other etiologies. Westeneng et al[19] analyzed aqueous humor samples of 56 immunocompromised patients using PCR. Out of these, 77 % of patients had infectious posterior or panuveitis. Forty nine percent patients had Cytomegalo virus (CMV), 7 % had Varicella Zoster virus (VZV), 26 % had T. gondii, 14 % had Treponema pallidum and 2 % each had Aspergillus and Candida. Viral infections were detected by PCR in 94 % of cases, while using Goldmann-Witmer coefficient (GWC) could detect the same in only 18 % of cases. However PCR detected T. gondii in only 40 % patients, while using GWC helped detect T. gondii in 90 % of cases.[19]

Knox et al[15] performed PCR on aqueous or vitreous samples of 38 eyes of 37 patients with diagnostic dilemmas in posterior uveitis. Of these cases, a definitive diagnosis of a viral infection could be made by PCR in 25 eyes. The PCR negative cases were ultimately diagnosed to be toxoplasmosis, syphilis, Behcet disease, fungal endophthalmitis or idiopathic inflammation. Thus both positive and negative PCR results had diagnostic significance in the study.

In cases with viral retinitis, PCR can provide specific etiological diagnosis. Tran et al [18] performed PCR on aqueous samples in 22 patients with necrotizing herpetic retinitis. Viral DNA was detected in 86.4 % of patients. Epstein-Barr virus (EBV) seroconversion was noted in one additional patient. In 19 patients with acute retinal necrosis (ARN), VZV was identified in 6 patients, herpes simplex virus 1 (HSV-1) in 2 patients, herpes simplex virus (HSV-2) in 4 patients and CMV in 4 patients. In 3 patients with progressive outer retinal necrosis (PORN), VZV was detected in all patients. None of the samples showed more than one virus.

The newer variants of PCR have made the diagnosis of active infectious etiology more accurate. Sugita [21] et al assayed 100 ocular fluid samples from patients with uveitis by using multiplex PCR (qualitative) and real-time PCR (quantitative), for human herpes virus (HHV) DNA (HHV1-8). Out of 16 patients with ARN, HSV-1 was detected in 2 cases, HSV-2 in 3 cases and VZV in 11 cases using multiplex PCR. In all 16 cases high copy numbers of viral DNA were noted using real-time PCR, indicating active viral replication. VZV was detected in 10 patients. EBV-DNA was detected in 19 samples (17 %) using multiplex PCR. However real-time PCR showed that only 6 out of the 19 samples showed high copy numbers. CMV was detected in 4 patients. One patient with unilateral, severe panuveitis showed high copy number of HHV6-DNA. Thus clinical relevance of virus infection can be evaluated by real-time PCR.

Dworkin et al[22] analyzed aqueous and vitreous samples from patients with posterior uveitis. They also concluded that through the use of real-time PCR, one may be able to distinguish false-positive results from true-positive results by comparison of viral load. Their study suggested that for suspected VZV or HSV infection, viral titers of less than 10 pathogens per microlitres may be false positive. Conversely, very high pathogen loads are more likely to be associated with active disease.

Aqueous humor analysis for diagnosis of posterior uveitis is a recent important addition in the investigations for posterior uveitis. Rothova et al [20] studied aqueous samples of 152 eyes with active posterior uveitis. Out of these 44 patients (29 %) tested positive for an infectious cause. 14 % of these were diagnosed by PCR only while 20 % were diagnosed by both PCR and GWC. PCR was more

informative in immunosuppressed patients and positive PCR results were observed more frequently for viral infections.

PCR is especially of importance in diagnosis of posterior uveitis with media opacity, which makes clinical diagnosis difficult. Mitchell et al[17] developed PCR primers with sensitivity of 93 % and specificity of 98 % for detection of CMV retinitis. Of the nine patients tested, 4 tested positive for CMV and 3 for VZV. The remaining two were subsequently judged to have toxoplasmosis. In all cases, the clinical course was consistent with the PCR-based diagnosis.

PCR has been shown to be very useful in linking particular pathogens to specific uveitic entities. Yamamoto et al [23] showed HSV as a possible cause in Posner-Schlossman syndrome with PCR being positive for HSV in all 3 cases with active glaucomatocyclitic crisis. Priya et al [48] analysed aqueous humor samples of nine cases with diagnosed serpiginous choroiditis. VZV and HSV DNA were detected in five and one patient respectively, suggesting these viruses as a possible cause in serpiginous choroiditis.

Clinical diagnosis of atypical toxoplasmosis can be problematic. Initial studies of PCR diagnosis of T. gondii were disappointing, showing sensitivity less than 50 %. [24, 25] However, recent advances in primer design, have greatly improved yields for PCR of T. gondii. Montoya et al [26] were able to detect Toxoplasma DNA in nearly 80 % of patients with suspected ocular toxoplasmosis and positive serum IgG titers. Bou et al [27] were able to detect T. gondii DNA in the peripheral blood of most patients with active ocular toxoplasmosis, raising the possibility that in the future, reactivation disease could be diagnosed via a blood test. Mahalakshmi et al [28] showed a positive PCR (nPCR) in 59.1 % of cases with clinically suspected ocular toxoplasmosis, which was not significantly less than Witmer Desmont's coeffiecient (WDC) (72.7 %). However, PCR was more acceptable because, amount of specimen required, time and cost was less than WDC. Also PCR gives direct evidence of T. gondii DNA in intraocular fluids.

Biswas et al [29] performed PCR on the aqueous in a case of suspected miliary tuberculosis of choroid and Mycobacterium tuberculosis genome was found in PCR analysis. PCR is also helpful in detecting Leptospira related uveitis. [30]

Diagnosis of retinal vasculitis

Madhavan et al [31] performed PCR on epiretinal membrane (ERM) specimens obtained from 23 patients with Eales' disease. Eleven out of 23 (47.8 %) were positive for Mycobacterium tuberculosis genome, indicating association of this bacterium with Eales' disease. Gupta et al [32] reported tubercular retinal vasculitis with varied fundus findings, and diagnosis was confirmed by doing PCR from the aqueous or vitreous humor.

Diagnosis of noninfectious uveitis

PCR has also been utilized in studies of noninfectious uveitis, HLA typing being the most common application. Saiki et al used PCR to amplify a specific segment of beta-globin or HLA-DQ genome in human DNA. [33] Shino et al reported complete association of HLA-DRB104 and DQB104 with Vogt-Koyanagi-Harada (VKH) disease. [34] PCR has also been used for HLA-B51 typing in Behcet disease[35] and HLA-B27 [36] typing in anterior uveitis. Intraocular cytokines and other inflammatory mediators have been identified via reverse transcription PCR. [37]

Diagnosis of masquerade syndrome

Masquerade syndrome is a group of disorders, most commonly malignancies, which mimic uveitis. PCR can be useful to differentiate them from true cases of uveitis. B-cell lymphoma in the eye, mimicking chronic uveitis, has been diagnosed using PCR. [38]

Diagnosis of endophthalmitis

Although direct microscopy is the easiest and most rapid method to detect bacterial etiologies of endophthalmitis, its sensitivity is very low, with positive results varying from 4.2 % to 46.5 % for vitreous samples, which decreases further in aqueous fluid.[39, 40]

More sensitive than microscopy, culture is considered the "gold standard". However the organisms are frequently present in low numbers, resulting in yields from diagnostic vitreous biopsies to less than 50 %. The Endophthalmitis Vitrectomy study (EVS) reported culture yields of only 70 %. [41]Culture results are also slow to return, requiring patients to be treated with broad-spectrum antibiotics for

several days even for relatively indolent bacteria.

PCR with its high sensitivity and specificity would be an ideal method in these cases. By using eubacterial and panfungal genome primers, the presence of the organisms in biopsy material from eyes with suspected endophthalmitis can be confirmed using PCR and that too within few hours. Therese et al [40] demonstrated the utility of this approach in culture-negative endophthalmitis. They were able to determine a bacterial cause for endophthalmitis in 100 % of culture-positive and 44 % of culture-negative cases. Of the remaining culture-negative cases, one third was found to have fungal etiology.

Lohmann et al [42] used eubacterial and panfungal primers, along with culture and stain for 25 eyes with delayed-onset endophthalmitis. Aqueous culture and microscopy each had 0% yield, but vitreous culture had a yield of 24 % in these cases. PCR of the aqueous yielded a diagnosis in 84 % of the cases and of the vitreous yielded a diagnosis in 92 %.

Biswas at al [43] demonstrated Aspergillus fumigatus fungus from paraffin section of an eyeball of an eight month old child removed for endogenous endophthalmitis. Compared to the conventional technique, PCR was found to be more rapid and sensitive method for detection of fungal DNA in postoperative fungal endophthalmitis. [44, 45, 46]

PCR thus has clear superiority to any other available

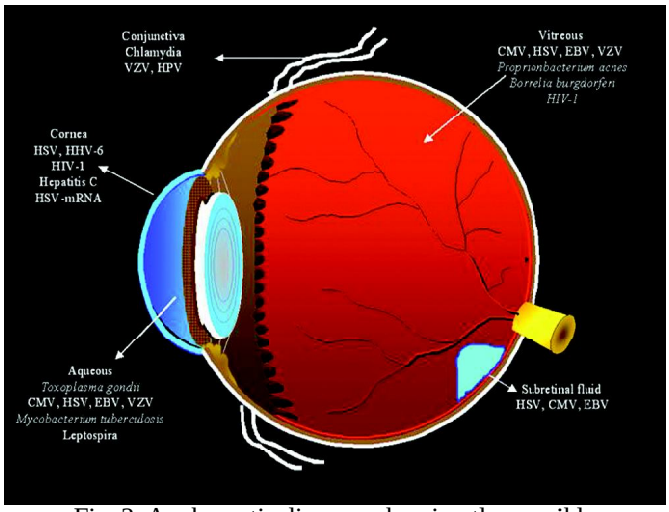
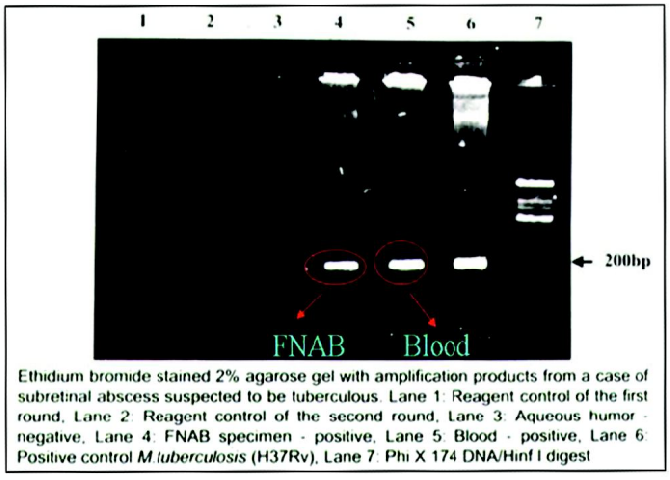


Fig. 2: A schematic diagram showing the possible sites for samples for PCR and the common etiological agents for each site/sample.



Ethidium bromide stained 2% agarose gel with amplification products from a case of subretinal abscess suspected to be tuberculous. Lane 1: Reagent control of the first round, Lane 2: Reagent control of the second round, Lane 3: Aqueous humor - negative, Lane 4: FNAB specimen - positive, Lane 5: Blood - positive, Lane 6: Positive control *M.tuberculosis* (H37Rv), Lane 7: Phi X 174 DNA/Hinf I digest

Fig. 3b PCR report of the aqueous humor, FNAB specimen and blood samples from the same patient

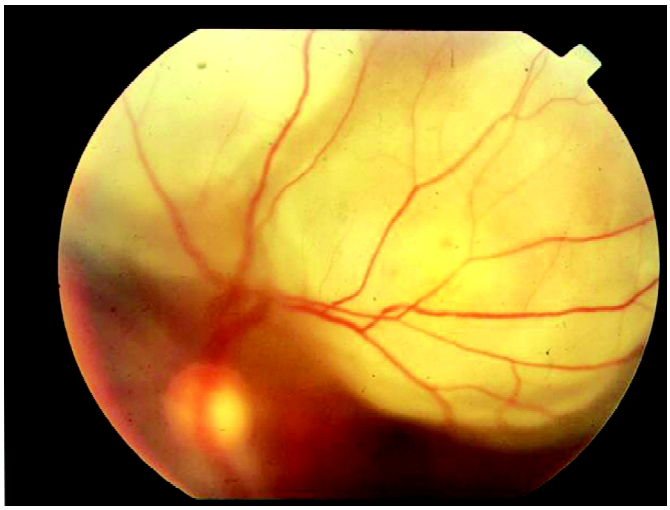


Fig. 3a: Pre-treatment fundus photograph of a case of subretinal abscess, suspected to be tuberculosis

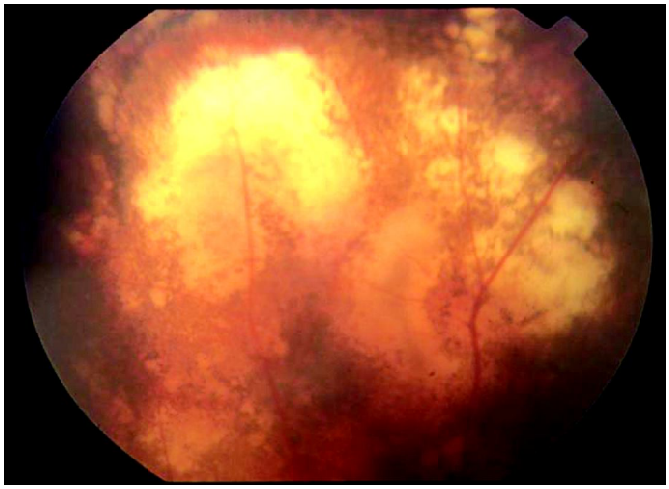


Fig. 3c Post-treatment photograph of the same patient showing complete resolution of subretinal abscess

diagnostic technique for diagnosis of endophthalmitis. It is also a useful adjunct to conventional culture because when used with aqueous humor samples only, the association of both techniques allowed for a microbiological diagnosis in 71 % of cases of postoperative acute and delayed-onset endophthalmitis.[47]

CONCLUSION

PCR is a powerful molecular technique for evaluation of very small amounts of DNA and RNA. PCR can be a simple, rapid, sensitive and specific tool for the diagnosis of intraocular inflammations of various etiologies.

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