Development and application of multiplex polymerase chain reaction for the etiological diagnosis of infectious endophthalmitis

Bagyalakshmi R, Madhavan HN, Therese KL

Larsen & Toubro Microbiology Research Centre, Vision Research Foundation, No.18, College Road, Sankara Nethralaya, Chennai - 600 006, Tamil Nadu, India

Correspondence:
H. N. Madhavan,
E-mail: drhnm@snmail.org

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ABSTRACT

Background: Uniplex polymerase chain reaction (PCR) for detection of bacterial and panfungal genome has been applied onto a large number of intraocular fluids facilitating management of infective endophthalmitis.

Aim: To develop and apply a novel, rapid multiplex polymerase chain reaction (mPCR) to detect the presence of eubacterial, *Propionibacterium acnes* and panfungal genomes in intraocular fluids from patients clinically diagnosed to have infective endophthalmitis.

Settings and Design: Prospective study.

Materials and Methods: Conventional methods of direct microscopy by KOH/calcofluor mount, Gram's staining and culture were done on 30 (19 Aqueous humor-AH and 11 Vitreous fluid-VF) intraocular specimens and mPCR done for simultaneous detection of eubacterial, *P. acnes* and panfungal genomes.

Results: mPCR detected an infectious etiology in 18 (60%) of 30 intraocular specimens. Eubacterial genome was detected in 12 (40%) specimens, *P. acnes* genome in 4 (13.3%) specimens and panfungal genome in 2 (6.6%) specimens. mPCR results correlated with those of uniplex PCR. mPCR results were available within 5-6 hours after receipt of specimen, as against 8 hours required for each uniplex PCR with three separate thermalcyclers for their completion. Consumption of *Taq* polymerase was reduced considerably for mPCR.

Conclusion: mPCR is a cost effective, single tube method for the simultaneous detection of eubacterial, *P. acnes* and panfungal genomes in intraocular specimens from patients with infective endophthalmitis. It is a more rapid procedure than uniplex PCRs and requires only a single thermalcycler.

KEY WORDS: Multiplex PCR, polymerase chain reaction, aqueous humor, vitreous fluid, infectious endophthalmitis.

Infective endophthalmitis is a serious ocular infection that can result in blindness. Approximately 70% of cases occur as a direct complication of intraocular surgery.[1,2] The diagnosis of infective endophthalmitis is on clinical grounds; but negative cultures are frequently encountered, (21-63%)[3] resulting in a dilemma. The results of conventional methods of direct smear are available within half an hour and, culture results are available at 48 hours (for bacteria) and at 10 days (for fungus and anaerobic bacteria). In contrast, polymerase chain reaction (PCR) results are available within 8 hours and hence PCR had proved to be a rapid, reliable and sensitive tool in diagnosis of infective endophthalmitis. multiplex polymerase chain reaction (mPCR) has been widely used for detection and characterization of genes of bacteria[4-6] and viral retinitis.[7] It is important to have a rapid and sensitive test that would help resolve the dilemma by detecting the genomes of common infectious agents simultaneously.

Materials and Methods

Thirty intraocular specimens (19 AH and 11 VF), collected from 25 patients referred to an ophthalmic hospital, during June-August 2004 with clinical diagnosis of infective endophthalmitis, after cataract or lens surgeries were investigated for detection of the causative infectious agent.

Conventional microbiological investigations

Both AH and VF were processed for KOH /calcofluor and Gram staining techniques and for culture of bacteria and fungi by standard microbiological methods.[8-10] In brief, the intraocular specimens were inoculated onto blood agar (incubated aerobically at 37ºC), chocolate agar (incubated in 10% CO_2 at 37ºC), brucella blood agar (incubated anaerobically in Don Whitley Compact anaerobic work station, Thane, India), brain heart infusion broth and thioglycollate broth. Sabouraud's

dextrose agar was used for isolation of fungus, and other aerobic media with no growth at the end of 48 hours were incubated for a period of 10 days to isolate fungus. The isolated microorganisms were identified by standard protocols. The smears made from AH and VF using the cytospin (Cytospin 2, Shandon, USA) were stained by Gram staining and KOH / calcofluor preparations for the detection of bacteria and fungus respectively.

PCR assay conditions for detection of eubacterial, P. acnes and panfungal genomes

DNA was extracted from the intraocular specimens of AH and VF by Qiagen kit (Qiagen, Germany, catalogue 51304) method according to the manufacturer's instructions. nPCR was carried out using eubacterial primers targeting for 16SrRNA[2] and panfungal genome targeting 28SrRNA[11] as described previously.

Assay conditions for mPCR

For a 50 µl reaction, 8 µl of 200 µm dNTPs, 5 µl of 10 x PCR buffer (5 mM $MgCl_2$, 500 mM KCl, 100 mM Tris Cl, (0.01% gelatin), 0.36 micromole of primers for eubacterial genome: U_1 5' TTGGAGAGTTTGATCCTGGCTC 3', rU_4 5' GGACTACCAGGGTATCTAA 3' (first round) U_2 5' GGCGTGCTTA ACACATGCAAGTCG 3', U_3 5' GCG GCTGGCACGTAGTTAG 3' (second round), 1 micromole of *P. acnes* primers Pa_1: 5' AAGGCCCTGCTTTTGTGG 3' rPa_3 5' ACTCACGCTTCGTCACAG 3' (first round) and Pa_1 and rPa_2 5' TCCATCCGCAACCGCCGAA 3' for the second round were used. For panfungal genome detection, 10 picomoles of forward primer FU_1 5' TGAAATTGTTGAAAGGGAA 3' and reverse primer FU_2 - 5' GACTCCTTGGTCCGTGTT 3' were used. The primers and PCR reagents were obtained from Bangalore Genei, India Amplification of the three genomes was carried out in a single tube using 10 µl of template DNA in Perkin Elmer thermalcycler (Model 2700) with the same thermal profile of Therese *et al*[2] for 25 cycles. Nested amplification for detection of eubacterial and *P. acnes* genomes was carried out with the same thermal profile for 10 cycles.

Sensitivity and specificity

Sensitivity of mPCR was determined using serial ten-fold dilutions of DNA of *Staphylococcus aureus* (ATCC 12228), of a laboratory isolate of *P. acnes* and of *C. albicans* (ATCC 24433); specificity of mPCR assay was determined using standard ATCC strains of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa*, (ATCC 7853), *Staphylococcus aureus* (ATCC 12228), *Mycobacterium tuberculosis* H37Rv, HSV 1 ATCC 733 VR and human leukocyte DNA.

Results

Sensitivity and specificity of mPCR

The specificity and sensitivity of both the nPCR tests for detection of eubacterial and *P. acnes* genome were 40 fg and 50 fg as published previously. The sensitivity of multiplex PCR for detection of eubacterial genome and *P. acnes* genome was 100 fg and for panfungal genome was 0.4 pg. The primers were specific when amplified by multiplex reaction amplifying the respective targets. The results of sensitivity of mPCR after first round of amplification are shown in Figure 1A and after second round of amplification are shown in Figure 1B.

The results of mPCR for detection of eubacterial, *P. acnes* and panfungal genomes are given in Table 1. The results of individual eubacterial PCR is shown in Figure 2 and that of

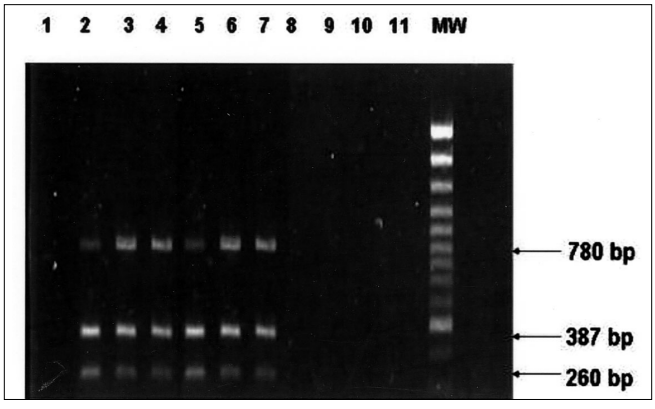

Figure 1A: Agarose gel electrophoretogram (2% agarose with ethidium bromide) showing the sensitivity of multiplex PCR using eubacterial, *P. acnes* primers targeting 16SrRNA, and panfungal primers targeting 28SrRNA after first round of amplification. Lane 1: Negative control I round Lane 2: Positive control : *S. aureus* (ATCC 12228), laboratory isolate of *P. acnes* and *Candida albicans* (ATCC 24433) Lane 3 – Lane 7 Serial 10 fold dilutions of Positive control DNAs MW : Molecular weight marker 100 bp DNA ladder

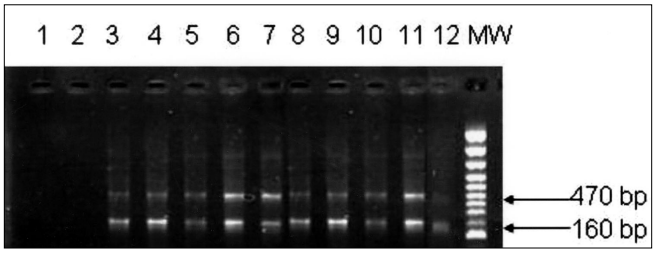

Figure 1B: Agarose gel electrophoretogram (2% agarose with ethidium bromide) showing the sensitivity of multiplex PCR using eubacterial, *P. acnes* primers targeting 16SrRNA after second round of amplification. Lane 1: Negative control II round Lane 2: Lane 1: Negative control I round Lane 3: Positive control : *S. aureus, P. acnes* Lane 4 – Lane 12: Serial 10 fold dilutions of Positive control DNA MW : Molecular weight marker 100 bp DNA ladder

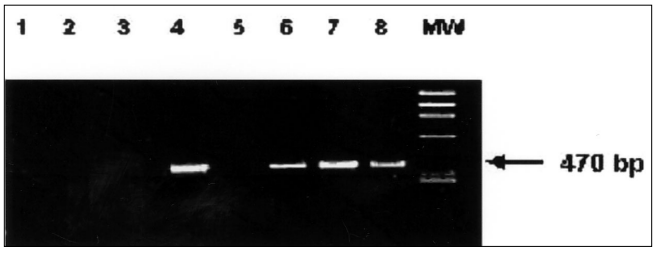

Figure 2: Agarose gel electrophoretogram (2% agarose with ethidium bromide) showing the results of nested PCR applied on intraocular specimens using eubacterial primers targeting 16SrRNA. Lane 1: Negative control II round Lane 2: Negative control I round Lane 3: Extraction control Lane 4: AH positive Lane 5: VF negative Lane 6 & Lane 7: VF positive Lane 8: Positive Control DNA (*S. aureus* ATCC 12228 strain) MW : Molecular weight marker Phi X 174 DNA / Hinf I digest

Table 1: Comparison of results of conventional methods and mPCR applied on intra-ocular specimens

M. No.	Intra-ocular specimen	Clinical diagnosis	Smear	Culture	Results of mPCR		
					Eubacterial	*P. acnes*	Panfungal
2667/04	AH	P.O.E	GPC	*S. epidermidis*	Positive	Negative	Negative
3294/04	VF	P.O.E	Negative	Negative	Positive	Negative	Negative
3081/04	AH	P.O.E	GPC	*S. aureus*	Positive	Negative	Negative
3078/04	VF	P.O.E	GNB	*Pseudomonas aeruginosa*	Positive	Negative	Negative
2839/04	AH	Chronic P.O.E	Negative	Negative	Positive	Negative	Negative
3486/04	VF	P.O.E	Negative	Negative	Positive	Negative	Negative
3892/04	VF	P.O.E	Negative	Negative	Positive	Negative	Negative
2863/04	VF	P.O.E	Negative	Negative	Positive	Negative	Negative
2722/04	AH	P.O.E	Negative	Negative	Positive	Positive	Negative
2801/04	AH	P.O.E	Negative	Negative	Positive	Positive	Negative
3955/04	AH	Delayed P.O.E	Negative	Negative	Positive	Positive	Negative
2837/04	AH	Chronic P.O.E	Negative	Negative	Positive	Positive	Negative
3532/04	AH	Post traumaticendoph	Negative	Negative	Negative	Negative	Positive
1439/04	VF	Endog.endoph	Negative	Negative	Negative	Negative	Positive

AH: aqueous humor, VF: vitreous fluid; P.O.E.: Post – operative endophthalmitis; Post- traumatic endoph: Post traumatic endophthalmitis; Endog. Endoph: Endogenous endophthalmitis; GPC: Gram positive cocci; GNB: Gram negative bacilli.

Propionibacterium acnes PCR is shown in Figure 3. mPCR revealed the presence of eubacterial genome in 12 [(7 AH, 5 VF) 40%], in additional 9 specimens increasing the clinical sensitivity by 30%. The results of mPCR applied on intraocular specimens for eubacterial genome detection is shown in Figure 4. mPCR revealed the presence of *Propionibacterium acnes* genome in 4 (4 AH,13.3%).The results of mPCR applied on intraocular specimens for *P. acnes* genome detection is shown in Figure 5. mPCR revealed the presence of panfungal genome in 2 (1 AH, 1 VF 6.3% [Table 1]). The results of multiplex PCR applied on intraocular specimens for panfungal genome detection is shown in Figure 6. There was no striking difference in the clinical presentation of cases detected by conventional methods as against those detected only by mPCR

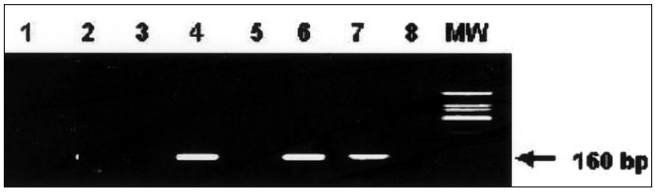

Figure 3: Agarose gel electrophoretogram (2% agarose with ethidium bromide) showing the results of nested PCR applied on intraocular specimens using *P. acnes* primers targeting 16SrRNA. Lane 1: Negative control II round Lane 2: Negative control I round Lane 3: Extraction control Lane 4: AH positive Lane 5: VF negative Lane 6: AH positive Lane 7: Positive Control DNA (*Propionibacterium acnes*) MW : Molecular weight marker Phi X 174 DNA / Hinf I digest

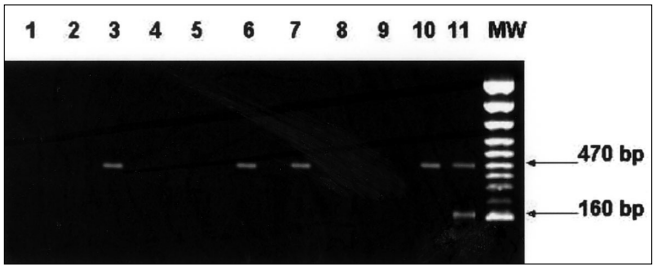

Figure 4: Agarose gel electrophoretogram (2% agarose with ethidium bromide) showing the results of multiplex PCR applied on intraocular specimens using Eubacterial, *P. acnes* and panfungal primers. Lane 1: Negative control II round Lane 2: Negative control I round Lane 3: AH positive Lane 4: VF negative Lane 5: VF negative Lane 6: VF positive Lane 7: AH positive Lane 8: VF negative Lane 9: AH negative Lane 10: AH positive Lane 11: Positive Control DNAs (*S. aureus, Propionibacterium acnes*) MW : Molecular weight marker 100 bp ladder

Discussion

In the present study, novel mPCR was developed and applied on to intraocular specimens to detect three infectious genomes. mPCR was evaluated against individual uniplex PCR and results were on par with them. mPCR has several advantages over individual PCR: It is cost-effective as it reduces the total cost by one-third, offers considerable

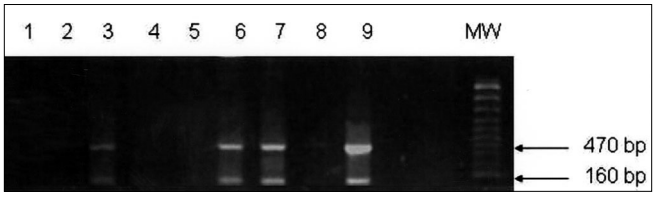

Figure 5: Agarose gel electrophoretogram (2% agarose with ethidium bromide) showing the results of multiplex PCR applied on intraocular specimens using Eubacterial, *P. acnes* and panfungal primers. Lane 1: Negative control II round Lane 2: Negative control I round Lane 3: AH eubacterial positive, *P. acnes* positive Lane 4: AH Negative Lane 5: VF negative Lane 6,7: AH positive for eubacterial and *P. acnes* Lane 8: AH Negative Lane 9: Positive Control

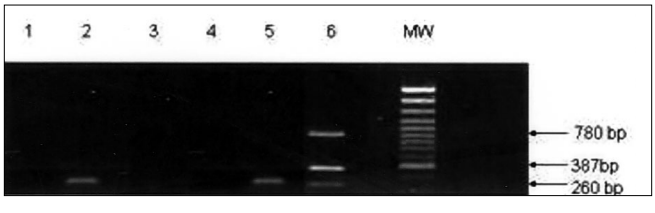

Figure 6: Agarose gel electrophoretogram (2% agarose with ethidium bromide) showing the results of multiplex PCR applied on intraocular specimens using Eubacterial, *P. acnes* and panfungal primers. Lane 1: Negative control I round Lane 2: AH positive Lane 3: VF negative Lane 4: AH negative Lane 5: VF positive Lane 6: Positive control (*S. aureus, Propionibacterium acnes, C. albicans*) MW: Molecular weight marker 100 bp ladder

reduction in time required for generating reports and eliminates the need to use separate thermalcyclers for individual uPCR. The consumption of PCR reagents is minimized by carrying out the amplification in a single tube with no additional *Taq polymerase* required to amplify three genomes simultaneously. The annealing temperature for mPCR was determined based on the melting temperature, length (18-24 bases) and GC content of the primers. The annealing temperature of 60°C was optimal for amplifying all three infectious genomes. This novel thermal profile was designed to amplify the three infectious genomes by using 25 cycles for the first round and a reduction of five cycles for the second round without affecting the sensitivity and specificity of the procedure. Moreover, the time period needed for reporting the results was reduced by two hours compared to individual PCRs. By application of mPCR rapid diagnosis was available within five hours of specimen collection as against 8 hours required for each uPCR. Furthermore, the results of mPCR correlated well with intraocular specimens which were culture positive for bacteria. These findings are comparable to our earlier findings[2] and as well as to those published by Lohmann *et al*, Hykin *et al* and Carroll *et al*.[11-14] This novel mPCR was extremely useful in diagnosing the infectious agent in the minimal amount of template DNA available. Moreover, this novel mPCR has several advantages over uPCR such as, detection of two or more targets in a single tube, minimizing the use of PCR reagents, reduction in time and the need for a single thermalcycler for amplification. Based on the results of mPCR appropriate therapy was initiated using antibacterial or antifungal drugs as the case may be. The newer technique of mPCR was extremely useful in management of endophthalmitis. To the best of our knowledge, this study is the first of its kind developed for etiological diagnosis of infective endophthalmitis.

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