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A study on the characterization of *Propionibacterium acnes* isolated from ocular clinical specimens

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***Background & objectives*: There are only a few reports available on characterization of *Propionibacterium acnes* isolated from various ocular clinical specimens. We undertook this study to evaluate the role of *P. acnes* in ocular infections and biofilm production, and also do the phylogenetic analysis of the bacilli.**

***Methods*: One hundred isolates of *P. acnes* collected prospectively from ocular clinical specimens at a tertiary care eye hospital between January 2010 and December 2011, were studied for their association with various ocular disease conditions. The isolates were also subjected to genotyping and phylogenetic analysis, and were also tested for their ability to produce biofilms.**

***Results*: Among preoperative conjunctival swabs, *P. acnes* was a probably significant pathogen in one case; a possibly significant pathogen in two cases. In other clinical conditions, 13 per cent isolates were probably significant pathogens and 38 per cent as possibly significant pathogens. The analysis of *16S rRNA* gene revealed four different phylogenies whereas analysis of *recA* gene showed two phylogenies confirming that *recA* gene was more reliable than *16S rRNA* with less sequence variation. Results of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) had 100 per cent concordance with phylogenetic results. No association was seen between *P. acnes* subtypes and biofilm production.**

***Interpretation & conclusions*: *RecA* gene phylogenetic studies revealed two different phylogenies. RFLP technique was found to be cost-effective with high sensitivity and specificity in phylogenetic analysis. No association between *P. acnes* subtypes and pathogenetic ability was observed. Biofilm producing isolates showed increased antibiotic resistance compared with non-biofilm producing isolates.**

Key words Biofilm - DNA-sequencing - genotyping - phenotyping - *Propionibacterium acnes* - restriction fragment length polymorphism

Propionibacterium acnes is a major normal resident of human skin[1]. *P. acnes* is an anaerobic to aerotolerant, non-spore-forming, pleomorphic Gram-positive bacillus with extremely slow growth characteristics on culture[2] and is found predominantly in the sebaceous gland-rich areas of the skin[3]. Apart from skin infections, *P. acnes* is associated with a wide range of ocular conditions such as dry eyes[4],

lacrimal sac and/or nasolacrimal duct obstruction[5], and ocular infections such as keratitis[6, 7], blepharitis[8], dacryocystitis[9], canaliculitis[10], and infections of prosthetic implants, including intraocular lenses (IOLs)[11]. *P. acnes* is responsible for devastating ocular complications due to infectious endophthalmitis; either as causative agent of endogenous[12] or as delayed[13] post-operative[14] or post-traumatic endophthalmitis[15].

Although numerous reports confirm the ability of *P. acnes* to produce vision-debilitating keratitis[6,7,16], yet in many routine diagnostic practices the clinical importance of *P. acnes* infections is underestimated. This is due to the lack of efficacy of routine detection and isolation procedures for identifying *P. acnes* as well as the traditionally-held view that *P. acnes* is a bacterium of low virulence, and its presence in clinical samples often is considered a contamination[17].

P. acnes is isolated more frequently (up to 8 times more) than are other species of *Propionibacterium*[18]. Two distinct phenotypes of *P. acnes* (types I and II), distinguished by serological agglutination tests and cell-wall sugar analysis, have been reported[19]. DNA sequence analysis of *P. acnes recA* gene has revealed types I and II genotypes which are phylogenetically distinct clusters or lineages. A new phylogenetic type, type III, that displays differences in cell surface antigen and cellular morphology has also been reported[20]. Several reports on the relationship of various genotypes of *P. acnes* from failed prosthetic hip-associated bone and tissue samples, as well as isolates from acne and dental infections, have been analyzed[17]. Genetic analysis of *P. acnes* to understand the phylogeny, and the association between the genotypic patterns and their role in ocular clinical infections has not yet been studied and needs to be understood.

Increasing prevalence of antimicrobial resistance among *P. acnes* has been documented in the last 20 years[21]. Despite susceptibility of *P. acnes* to various antibiotics, it is difficult to eradicate, and prolonged therapy is often recommended for biofilm-producing *P. acnes*[22]. On contact lenses, biofilm formation is believed to contribute to the development of microbial keratitis. Cataract surgeries with intraocular lens (IOL) placement or the introduction of intraocular infusion pumps, glaucoma tubes, stents, keratoplasties, or other ocular prostheses create opportunities for the development of infections involving microbial biofilms[23].

Though an association has been reported between *P. acnes* infection and development of keratitis and endophthalmitis, a detailed molecular characterization of *P. acnes* isolated from ocular clinical specimens has not been performed.

Therefore, the present study was done to understand the role of various phylogenetic types of *P. acnes* in ocular clinical infections and their relation with biofilm production which in turn reflects the degree of resistance/sensitivity of *P. acnes* to the various antibiotics used in the treatment.

Material & Methods

A total of 100 *P. acnes* isolates, 25 from pre-operative conjunctival swabs and the remaining 75 from other ocular clinical conditions (conjunctivitis, dacryocystitis, blepharitis, keratitis, endophthalmitis, scleritis, orbital cellulitis, orbital abscesses, graft infection, socket implant exposure) during a period of two years (January 2010 - December 2011), from a total of 7,598 ocular clinical specimens received for microbial culture at the Microbiology Research Centre, located in a tertiary care eye hospital Sankara Nethralaya, Chennai, Tamil Nadu, India, were included. Three American Type Culture Collection strains (ATCC, USA) namely, *Propionibacterium acnes* ATCC 11828, *Clostridium sporogenes* ATCC 11437 and *Bacteroides fragilis* ATCC 23745 were used as anaerobic quality control strains. The study protocol was approved by the institutional ethics sub-committee (IRB). Clinical specimens were processed as described elsewhere[24].

Anaerobic culture: *P. acnes* was identified based on conventional methods such as Gram staining, catalase, indole positivity, and growth characteristics in Brucella blood agar enriched with 5 per cent sheep blood, 5 mg/l haemin, 1 mg/l vitamin K1, (Hi-Media Laboratories Private Limited, Mumbai, India) after incubation in an anaerobic work station (Don Whitley Scientific Limited, West Yorkshire, UK) (85% N_2, 10% H_2, 5% CO_2).

Fermentation tests for differentiation of types of P. acnes: Fermentation reactions of *P. acnes* were studied on modified protease peptone yeast agar plates containing 40 mg of bromocresol purple indicator/litre and 1 per cent (wt/vol) sorbitol (Hi-Media Limited, Mumbai, India). Organisms were grown anaerobically, and a positive fermentation reaction was said to have occurred if agar plates turned yellow due to acid production[17].

Criteria for classifying P. acnes[18]:

(i) P. acnes as probably significant pathogen - *P. acnes* were acknowledged as probably significant

pathogen, if they are seen in the direct smear along with inflammatory cells and simultaneously isolated as the only microbial agent from the specimen along with abundant growth in culture.

(ii) P. acnes as possibly significant pathogen - *P. acnes* were recognized as a possibly significant pathogen, if they are seen in direct smear with moderate or scanty growth in culture at the site of inoculation of the specimen with another microbial agent.

(iii) P. acnes as an uncertain significant pathogen - *P. acnes* were identified as uncertain pathogen, when they are not seen in direct smear, yet isolated from site of inoculation from a single culture medium.

Molecular analysis of the P. acnes: All PCR reagents used for amplification including primers were procured from Merck, Darmstadt, Germany. The PCR amplifications were carried out using PCR thermal cycler Perkin Elmer Model 2700 (Applied Biosystems, USA). Sensitivity of all the uniplex PCRs ranged from 10 to 50 ng *P. acnes* ATCC DNA. The primers were highly specific, and no amplification was observed with fungal, viral, and human DNA.

(i) Species-specific PCR based DNA sequencing for identification of the *P. acnes* - To confirm the results of *P. acnes* identification obtained by phenotypic methods, PCR-based DNA sequencing was performed using *P. acnes* species-specific primers targeting the *16S rRNA* gene sequences as described earlier[25].

(ii) Amplification of 16S rRNA and recA genes to understand the phylogeny - DNA was extracted from a single colony isolated on Brucella sheep blood agar using the Qiagen DNA Mini kit (Qiagen, Germany) as per the manufacturer's instructions. The resulting genomic DNA was stored at -20°C until subjected to PCR analysis. The *16S rRNA* gene (1532 bp) was amplified using the primers described by Stubbs *et al*[26] and *recA* gene was amplified using a method described by McDowell *et al*[17]. Extracted DNA (5 µl) was added to 45 µl of PCR mixture consisting of 5 µl buffer (10× buffer containing 15mM $MgCl_2$), 200 µM dNTPs, 25 picomoles of primer, 30µl deionized water and 1.25U *Taq* polymerase. PCRs were carried out with the positive control containing DNA of *P. acnes* (ATCC 11828). A negative control was included in all the experiments. PCR products were analyzed by electrophoresis on 2 per cent agarose gel (Sisco Research Laboratories (SRL), Maharashtra, India) containing 1x tris-acetate-EDTA buffer along with molecular size markers (100 bp ladder). Resolved DNA products were stained with ethidium bromide, (50 ng/ml, Hi-Media, Mumbai, India) and viewed under UV light using gel documentation system (Vilber Lourmat, France).

(iii) Nucleotide sequence analysis - Purification of PCR products was performed by adding 1 µl of 1U/µl shrimp alkaline phosphatase (SAP) and 0.5 µl of 20 U/µl exonuclease (Exo) I (Fermentas, Life Science, USA) to 5µl of amplified product in a separate vial and then incubated at 37°C for 15 min followed by 85°C for 15 min. Exo-SAP-treated products were then subjected to cycle sequencing reaction as described earlier[27]. Products were purified according to standard protocol, loaded onto ABI PRISM 3130 DNA sequencer (Perkin-Elmer Applied Biosystems, USA) with polymer POP7 and sequenced. Sequences were analyzed using BIO EDIT, (*http://www.mbio.ncsu.edu/BioEdit/bioedit.html*), and finally blasted in (NCBI Blast website *http://blast.ncbi.nlm.nih.gov/Blast.cgi*) to identify species and DNA homology.

(iv) Phylogenetic analysis - The phylogenetic relationships of *P. acnes* were analyzed using *16S rRNA* and *recA* genomes using Data Analysis in Molecular Biology and Evolution (DAMBE) software (*http://web.hku.hk/_xxia/software/software.htm*) and MEGA (Molecular Evolutionary Genetics Analysis from: *http://www.megasoftware.net/index.html*). Multiple sequence alignments were performed by using the CLUSTAL W algorithm[28] and were exported into the DAMBE programme[29]. Phylogenetic trees were constructed by the maximum-parsimony method[29] and the neighbour-joining method[29]. The bootstrapping resampling statistics were performed using 100 data sets for each analysis.

(v) Designing, optimization and analysis of PCR - restriction fragment length polymorphism (RFLP) - For genotyping of *P. acnes* isolates, an appropriate restriction was found by subjecting known nucleotide sequence of 16S *rRNA* gene of *P. acnes* NCTC 737 (GenBank accession no.AY642055) and NCTC 10390 (GenBank accession no.AY642061) to Restrictionmapper.3 software (*http://www.restrictionmapper.org/*) which would identify the specific restriction enzyme from a panel of 200 enzymes to cleave the amplified product. Restriction enzyme "*Hin6I* (HinP1I-G/CGC)" possessed the ability to fragment 16S *rRNA* nucleotide sequences to determine the genotype. Reaction mixture contained Milli Q water-15 µl, Restriction buffer-4 µl, amplified product- 10 µl, and restriction enzyme-1 µl (1 U/µl, Fermentas Life Science, USA) and was

incubated for two h at 37°C after which entire digested sample was viewed in 3 per cent agarose gel.

Detection of biofilm production by tissue culture plate method (TCP): All 100 isolates from fresh Brucella sheep blood agar plates were inoculated into thioglycollate medium (Hi-Media Limited, Mumbai, India) and incubated anaerobically for 24 h at 37°C in a stationary condition and were diluted 1 in 10 with fresh medium. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates (Tarson, West Bengal, India) were filled with 200 µl aliquots of the diluted cultures. Sterile uninoculated thioglycollate medium served as a medium control to check sterility. *P. acnes* ATCC 11828, a non-biofilm producer, served as the inoculum control and negative control. A laboratory isolate of *P. acnes* with high drug resistance (isolated from eviscerated material) was used as positive control. Plates were incubated anaerobically for 18 to 24 h at 37°C.

At the end of the incubation period, the content of each well was gently removed by tapping the plates. The wells were washed four times with 200 µl of phosphate buffer saline (PBS *p*H 7.2, Hi-Media Limited, Mumbai, India) to remove free-floating 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed by addition of 200 µl sodium acetate (2%, Hi-Media Limited, Mumbai, India) and incubated at room temperature for 20 min[30]. After removing sodium acetate, crystal violet (200 µl, 0.1% w/v, Hi-Media Limited, Mumbai, India) was added and the plates were incubated for 20 min at room temperature. Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Subsequently, the crystal violet dye bound to adherent cells was released by adding 160 ml 33 per cent acetic acid (Sisco Research Laboratories Maharashtra, India).

Optical density (OD) of stained adherent bacteria was determined with a microELISA auto reader, (Bio-tek instruments, USA) at a wavelength of 520 nm. The OD values were considered as an index of bacteria adhering to surface and forming biofilms. Each experiment was performed in triplicate. To compensate for background absorbance, OD readings obtained for sterile medium, fixative solution, and dye were averaged and subtracted from test values. Mean OD value obtained from medium control was deducted from test values. The biofilm formation and adherence of *P. acnes* isolates were interpreted based on mean OD values[30]. The isolates were considered non-adherent and non or weak biofilm producers if mean OD value was <0.12, moderate with OD 0.12-0.24, and strongly adherent and high biofilm producers with OD >0.24.

Antimicrobiol susceptibility testing: All isolates were tested against antibiotics (µg/disc) used for clinical purpose namely ciprofloxacin (5), moxifloxacin (5), tobramycin (10) and for intravitreal injection of ceftazidime (30), cefazolin (5), amikacin (30 µg/disc), and vancomycin (30)[31]. All antibiotics were purchased from Hi-Media, Mumbai, India.

Minimum inhibitory concentrations (MICs) of antibiotics namely ciprofloxacin, norfloxacin, nalidixic acid, clindamycin, penicillin G, vancomycin and metronidazole (Sigma, USA), cefotaxime and imipenum (Ranbaxy, India) were measured by agar dilution technique as described by the Clinical Laboratory Standard Institute (CLSI)[32] with 10^5 cfu/spot in Brucella base sheep blood agar[33].

Statistical analysis: Probably significant *P. acnes* were compared with the possibly significant *P. acnes* with that of type I and type II using Fisher's exact test.

Results

Among these 100 isolates, *P. acnes* was recovered in pure culture from 38 specimens, predominantly from keratitis cases. In another 47 specimens, mostly from keratitis and conjunctivitis, *P. acnes* was isolated in addition to a single or multiple bacterial species and in the remaining 13, *P. acnes* was isolated with fungi. *P. acnes* were isolated along with other bacteria and fungus from two cases of keratitis following trauma.

P. acnes was isolated predominantly from conjunctival swabs (25/100, 25%) collected during preoperative procedure before cataract and trabeculectomy surgeries, followed by 25 isolates from keratitis (25%), 12 from dacryocystitis (12%), 10 from conjunctivitis (10%), eight from blepharitis (8%), five each from patients with inflammation due to recent history of trauma (broom stick, fire cracker, lemon tree thorn, insect bite and hammer, 5% each) and orbital abscesses (5%), four from endophthalmitis (4%), three others from corneal graft infection (3%), two from scleritis (2%) and one from cellulitis (1%).

P. acnes isolation rate: *P. acnes* was isolated as a probably significant pathogen in 14 patients (4 from keratitis and 3 from conjunctivitis, 2 from dacryocystitis and 1 each from blepharitis, scleritis, abscesses, graft infection and from pre-operative conjunctival swab. *P. acnes* was a possibly significant pathogen in 39

patients (14 from keratitis, 6 from blepharitis, 5 from trauma induced keratitis, 4 from conjunctivitis, 3 from endophthalmitis), and 2 from pre-operative cataract and trabeculectomy surgeries. *P. acnes* isolated as an uncertain significant pathogen in 47 patients, predominantly from routine preoperative conjunctival swabs (n=22) in the absence of suspension of infection and inflammation in the eye followed by decryocystitis (n=9) and keratitis (n=1).

Species specific PCR for confirming the phenotypic identification of P. acnes: One hundred isolates primarily identified by staining, biochemical and growth characteristics as *P. acnes* gave amplified products with the species specific uniplex PCR targeting *16S rRNA* gene for *P. acnes*. Fig. 1 shows the amplification of *P. acnes* from various specimens.

Species specific PCR for amplification of 16S rRNA of the P. acnes: Fig. 2 shows amplification of *16S rRNA* gene of *P. acnes* from various specimens. PCR products were subjected to RFLP and the remaining products were used for nucleotide sequence analysis to understand their phylogenetic origin.

Phylogenetic analysis:

DNA sequence analysis of *16S rRNA*: Amplified products of *16S rRNA* gene of all the isolates along with the *P. acnes* ATCC 11828 were subjected to DNA sequence analysis along with reference strains *P. acnes* NCTC 737 representing genotype I (GenBank accession no.AB042288), and NCTC 10390 representing genotype II (GenBank accession no.AY642044) to Multalin software (*http://multalin.toulouse. inra.fr/multalin/ multalin.html*). Genbank accession numbers of *16S rRNA* gene sequences of *P. acnes* were JF277163 - JF277165, JF289271 - JF289273, JF430007 - JF430010, JN700213 - JN700225, JN700227 - JN700231, JN700233 - JN700236, JN714989 - JN714997 (41 sequences).

PCR-RFLP analysis - PCR-RFLP protocol was standardized with the restriction enzyme *Hin6I*. *P. acnes* type specific polymorphism at 827th nucleotide position that corresponded to the nucleotide T in type I strains and C in type II strains (numbering corresponds to GenBank accession no. AB042288). The amplified products of *16S rRNA* gene of *P. acnes* when subjected

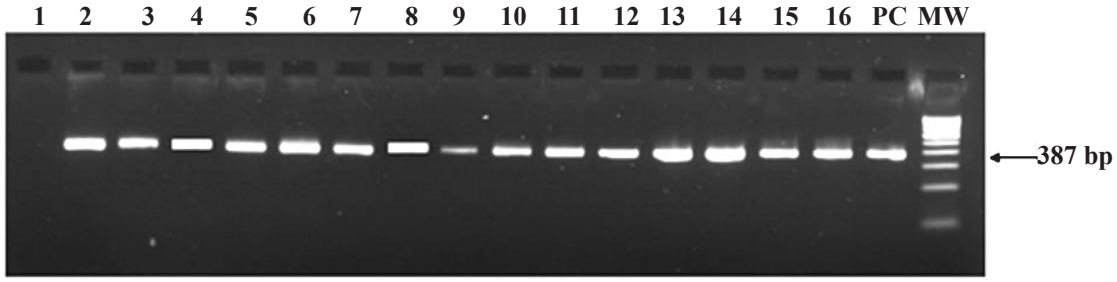

Fig. 1. Agarose gel electrophoretotogram showing results of uniplex PCR positive for *P. acnes* extracted from various isolates. Lane 1; Negative control; Lanes 2-16 showing positivity for *P. acnes*; Lane 17: PC (positive control) *P. acnes* (ATCC 11828); Lane 12: MW 100 bp ladders.

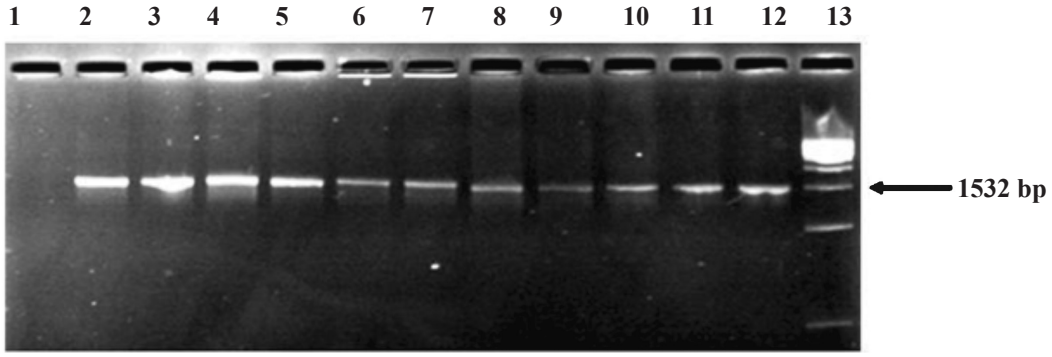

Fig. 2. Agarose gel electrophoretogram showing results of *16S rRNA* PCR amplified products of 10 *P. acnes* isolates. Lane 1: Negative control, Lanes 2-11: showing positive *P. acnes* isolates, Lane 12: PC (positive control) - *P. acnes* ATCC 11828, Lane 13: MW - 500 bp ladder.

to RFLP analysis yielded a total of four different RFLP patterns (type IA, B, C and type II). Based on type specific polymorphism at 827th position; patterns were divided as type I and type II. Type I isolates were further subdivided into types IA, IB, IC based on restriction digestion profile.

Restriction digestion profiles were as follows: *(i)* Type IA restriction pattern consisted of fragments 1085, 320 and 105 bp in size; *(ii)* Type IB restriction pattern consisted of fragments 670, 424 and 416bp in size; *(iii)* Type IC restriction pattern consisted of fragments sizes of 670, 424, 301 and 115 bp; and *(iv)* Type II restriction pattern consisted of fragments sizes of 670, 424, 256 and 160 bp.

Distributions of RFLP patterns observed among *P. acnes* isolates are shown in Table I. RFLP patterns were grouped based on band position in the 3 per cent agarose gel and were also verified by molecular weight analysis using the BioID software (Vilber Lourmat, Saint Jean de Braye**,** France).

Table I. Results of PCR-RFLP on *16S rRNA* gene PCR products of *P. acnes* isolates

Specimens	Types				
	No of isolates	I A	I B	I C	II
Preoperative conjunctival swab	25	1	7	8	9
Conjunctivitis	10	4	3	1	2
Blepharitis and dacryocystitis	20	-	10	7	3
Keratitis	29	-	17	11	1
Abscesses	5	-	0	5	-
Scleritis and cellulitis	3	-	2	1	-
Endophthalmitis	4	-	1	2	1
Implant/graft infection	4	-	3	1	-
Total	100	5	43	36	16

The predominant RFLP patterns observed in this study were type IB (46.0%) followed by type IC (36.0%) (Fig. 3). Type IA pattern was common only among conjunctival swabs; one from pre-operative and other four from conjunctivitis. Type IB pattern was exhibited predominantly in keratitis cases (n=17), followed by blepharitis and dacryocystitis (n=10). Type IC pattern was mainly seen in keratitis (n=11), followed by pre-operative conjunctival swabs (n=8), and blepharitis and dacryocystitis (n=7), Pre-operative conjunctival swabs (9%) exhibited mostly a type II RFLP pattern; one case each of traumatic keratitis and traumatic endophthalmitis also exhibited the type II RFLP pattern.

DNA sequence analysis of recA genes: All amplified products of *recA* gene were subjected to DNA nucleotide sequence alignment using Multalin software

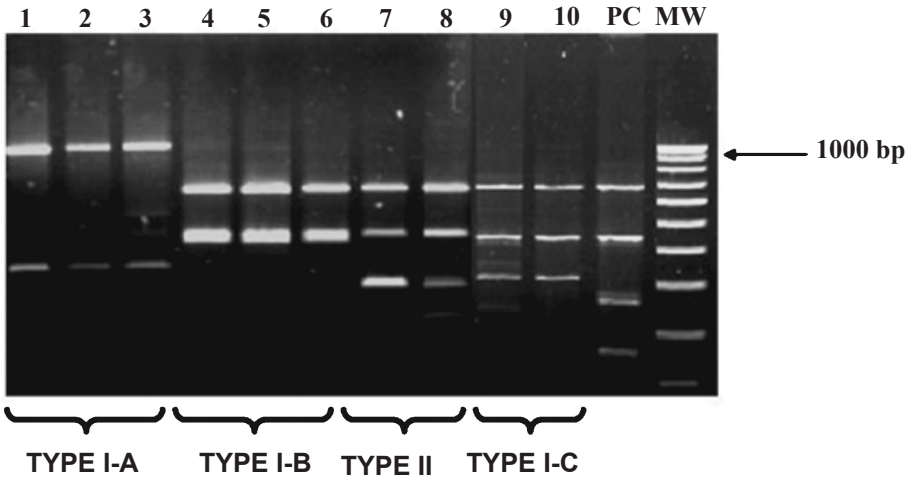

Fig. 3. Agarose gel electrophoresis of fragments produced by digestion of 1532 bp *16S rRNA* gene PCR amplification products of *P. acnes* predominantly isolates from conjunctival swabs. Lanes 1-3: Type I A isolates (Type I A : 1085, 320 & 105 bp); Lanes 4-6: Type I B isolates (Type I B : 670, 424 & 416 bp); Lanes 7-8: Type II isolates (Type II : 670, 424, 256 & 160); Lanes 9-10: Type I C isolates (Type I C : 670, 424, 301 and 115); PC: *P. acnes* ATCC 11828; MW: 100 bp ladder.

Table II. Results of PCR based sequencing of *recA* gene products of *P. acnes* isolates

Specimens	No. of isolates	Type I B	Type II
Cataract - Preoperative conjunctival swab	25	18	7
Conjunctivitis	10	7	3
Blepharitis and dacryocystitis	20	10	-
Keratitis	29	27	2
Abscesses	5	5	-
Scleritis and cellulitis	3	3	-
Endophthalmitis	4	3	1
Implant/graft infection	4	4	-
Total	100	87	13

along with the *P. acnes* ATCC 11828 and with reference strains *P. acnes* NCTC 737 and NCTC 10390. Results of PCR based sequencing of amplified *recA* gene PCR products of *P. acnes* isolates are shown in Table II. Genbank accession numbers of *recA* gene sequences of *P. acnes* isolates were JF836806, JF430011, JN864886, JN864888 - JN864912, JN864914, JN864915, JN864917 - JN864924, JN864926 - JN864935 (48 sequences).

Phylogenetic tree: Phylogenetic trees were constructed using amplified products of *16S rRNA* and *recA* gene sequences of *P. acnes* isolates having 99-100 per cent identity. Nucleotide sequences of both *16S rRNA* and *recA* genes obtained from *Helicobacter pylori* (GenBank accession no. U13756 and AE000615) and *Streptococcus agalactiae* (GenBank accession no. AF326345 and NC004116) were used as out groups for phylogenetic analysis. The consensus tree obtained by using the maximum-parsimony method for *16S rRNA* genome and for housekeeping gene *recA* (Figs. 4 and 5) revealed that both the *16S rRNA* and *recA* phylogenies of *P. acnes* were highly distinct from unrelated species selected as out groups for the trees (bootstrap values, 100%).

Phylogenetic trees of *16S rRNA* and *recA* genes based on protein translation of each nucleotide sequence revealed similar clustering of types I and II as distinct phylogenetic groups. Among the 100 isolates, those belonging to type II formed a single clade along with type II reference strain sequence, and the rest clustered along with type I *P. acnes* reference strains. Results of PCR-based RFLP techniques were in 100 per cent concordance with phylogenetic results.

A smaller number of genomic sequence variations were observed in the *recA* gene sequences, as compared to *16S rRNA* gene sequences, suggesting the *recA* gene a suitable marker in identification of phylogeny of ocular *P. acnes*.

Results of in vitro biofilm production: Among the 100 *P. acnes* isolates tested, a total of 16 isolates (16%) produced biofilm; seven isolates from conjunctivitis, one from traumatic keratitis and three isolates from eviscerated material obtained from infectious endophthalmitis showed abundant production of biofilm while four isolates from conjunctival swabs and one from eviscerated material (infectious endopthalmitis) exhibited moderate biofilm production (Fig. 6). None of the isolates collected from pre-operative swabs produced biofilm. Biofilm production versus MIC data showed that the high and moderate biofilm producing isolates were resistant to more than three antimicrobial agents compared with the isolates which lacked in the production of biofilm (Table III).

Antibiotic response: All patients showed clinical susceptibility to topical antibiotics namely ciprofloxacin, moxifloxacin, tobramycin and for intravitreal injection of ceftazidime, cefazolin, amikacin and vancomycin. As a pre-operative measure, before undergoing cataract surgery, topical antibiotic such as ciprofloxacin is usually given to control the antimicrobial load and, therefore, reducing the risk of post-operative infection. Amphotericin B, natamycin and fluconazole were used as antifungal agents in treatment of fungal co-infection with *P. acnes*.

Discussion

P. acnes is the predominant anaerobe isolated from ocular specimens[32]. The conventional method of detecting *P. acnes* was 100 per cent concordant with the uniplex PCR specific for *P. acnes*. In this study, the isolated *P. acnes* were predominantly from conjunctival swabs taken from outpatients or during preoperative procedure before performing cataract and trabeculectomy surgeries. Another important finding was that in 80 per cent of *P. acnes* isolated from corneal scraping fungus was isolated in culture. This emphasizes the ability of *P. acnes* to co-exist in

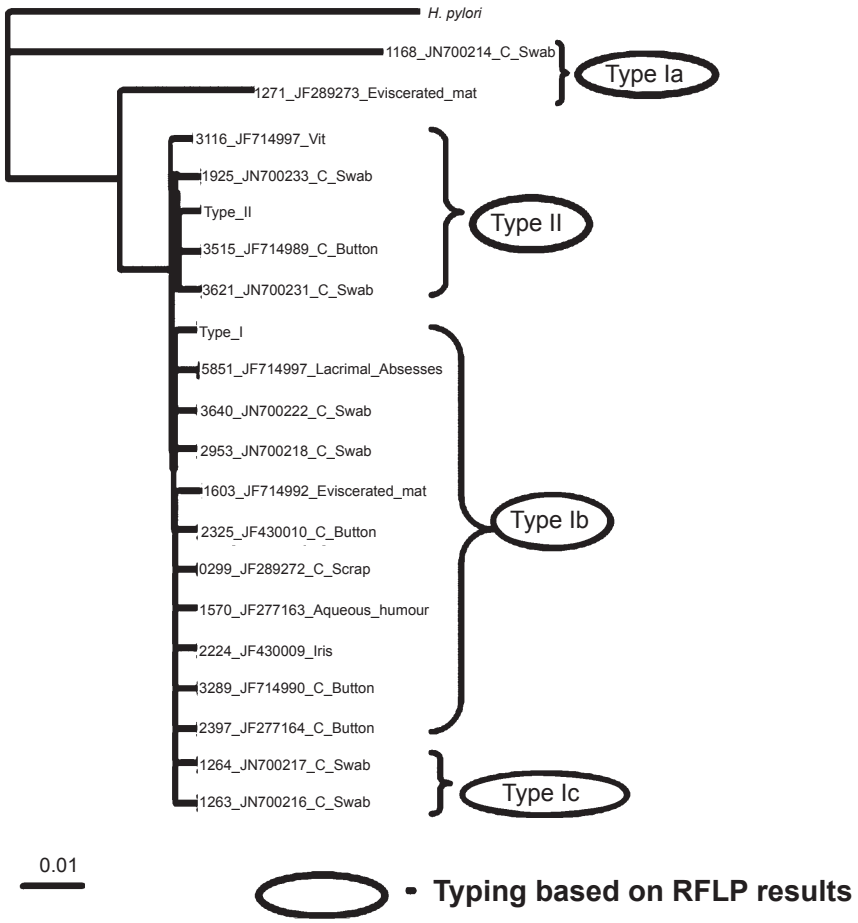

Fig. 4. Phylogenetic tree of *P. acnes* isolates based on the *16s RNA* gene sequences by maximum-parsimony method. Multiple sequence alignments were performed on these sequence products of genes and tree was constructed with the published sequences for *Helicobacter pylori* (U13756) and *Streptococcus agalactiae* (AF326345). Bootstrapping resampling statistics were applied to the trees (100 data sets). Phylogenetic analysis was carried out on a selection of isolates chosen to represent different nucleotide sequences.

fungal infectious keratitis. Polymicrobial infections were along with bacteria other than *P. acnes* and fungi were encountered in traumatic cases of keratitis and endophthalmitis.

Co-existence of *P. acnes* in blepharitis infections has been reported earlier[8]. In our study, *P. acnes* was detected in 11 cases of blepharitis. Similarly, *P. acnes* was isolated in association with dacryocystitis in 10 per cent of cases which was slightly higher than that reported by Brook and Frazier (9.4%)[9].

In a study conducted by Essex *et al*[15], 17.6 per cent of *P. acnes* isolates were shown to cause infection in post-traumatic patients. In our study, 5 per cent of *P. acnes* isolates were from trauma cases. Further, in 79 per cent cases, *P. acnes* was isolated without a previous history of infection or injury proving it to be a normal flora of the eye. However, in 21 per cent cases, *P. acnes* was isolated from infectious conditions. Our findings show that *P. acnes* though exists as normal flora in healthy eye, it can also be a causative agent of infection.

DNA based molecular techniques played a significant role in the phylogenetic characterization of ocular *P. acnes*. In our study, type I isolates were further typed into A, B, C based on restriction sites to *Hin6I* of *16S rRNA* gene amplified products. Theses subtypes were not specific to sites of collection of clinical specimens. Type III isolates were not encountered among the isolates tested. The identification of type-specific nucleotide differences between types I and II has revealed that DNA sequencing can be used as an accurate method for the identification of *P. acnes* types.

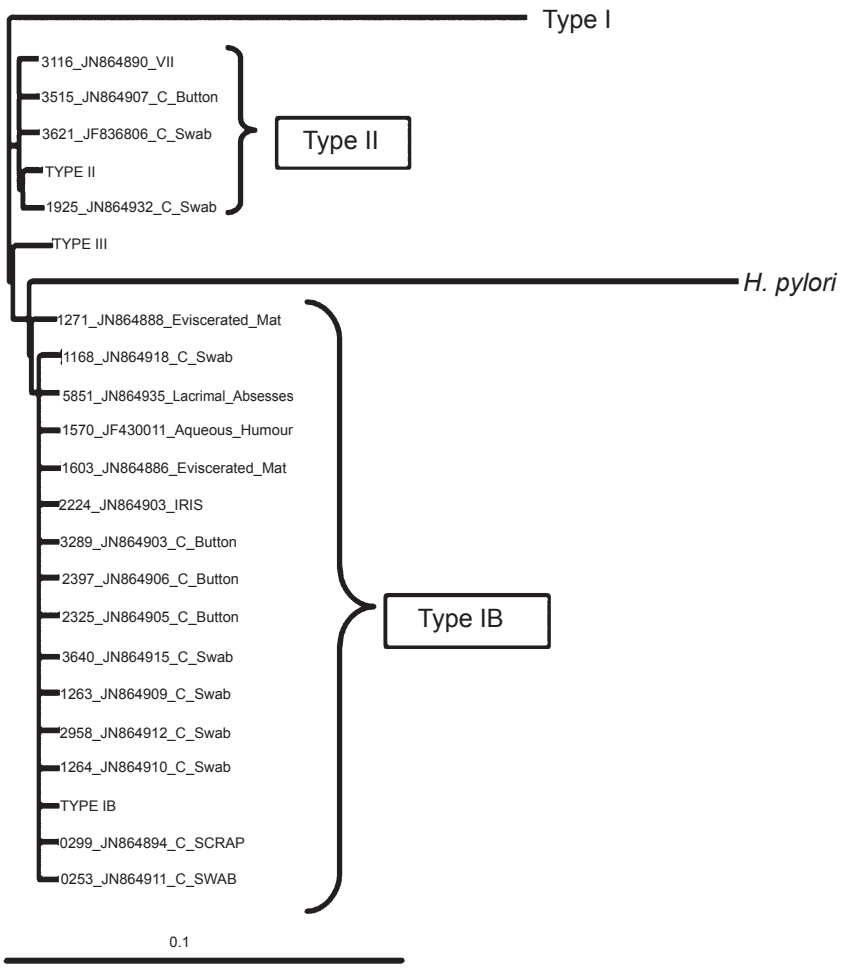

Fig. 5. Phylogenetic tree of *P. acnes* isolates based on the *recA* gene sequences by maximum-parsimony method. Multiple sequence alignments were performed on these sequence products of genes and tree was constructed with the published sequences for *Helicobacter pylori* (AE000615) and *Streptococcus agalactiae* (NC004116). Bootstrapping resampling statistics were applied to the trees (100 data sets). Phylogenetic analysis was carried out on a selection of isolates chosen to represent different nucleotide sequences.

Even though sequence analysis of *16S rRNA* gene is widely used for understanding the phylogenetic relationship between bacterial isolates, these are unsuitable when used to differentiate between related members of a genus or species which is replaced by a non-ribosomal housekeeping genes *recA*. The diversity and number of species from which sequences are available makes *recA* a potentially useful tool for molecular systematic studies of bacteria by giving better perception of phylogenetic relationship that exists between closely related organisms especially among *P. acnes* is proved by many studies[17,18,20].

The *16S rRNA* gene is considered to be the 'gold-standard' for investigating the phylogenetic relationship between bacterial organisms since these are highly conserved[20]; but inability of the same to distinguish the closely related ones pose the challenge. In our study *recA* gene, a protein-encoding genes with housekeeping functions, provided a better foundation for bacterial systematic differentiation of closely related organisms due to a higher neutral mutation rate within such genes.

Results of PCR-based RFLP on *16S rRNA* was 100 per cent in concordance with DNA sequencing results and fermentation experiments. The characteristic banding pattern was specific for type I and type II *P. acnes*. Therefore, RFLP technique is cost-effective and rapid compared with the DNA sequencing technique

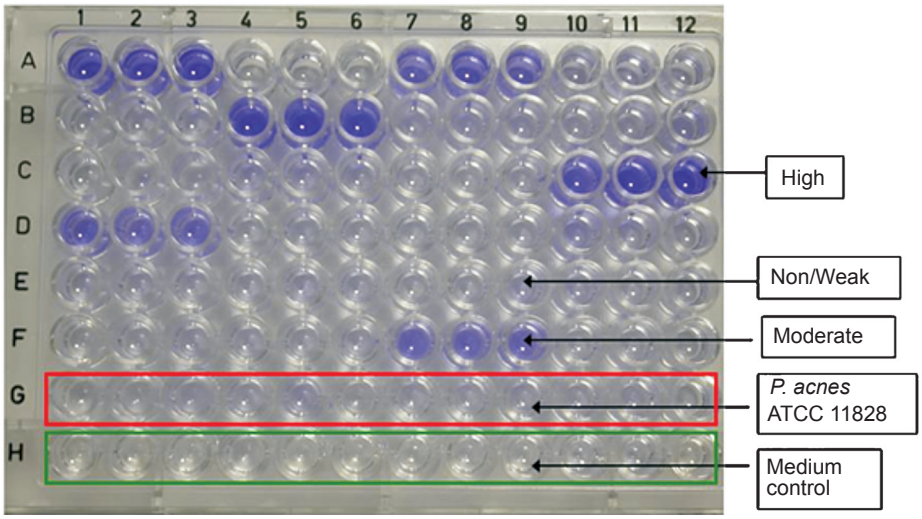

Fig. 6. Tissue culture plate showing the results of biofilm production: Row A1-A3, B4-6, C10-C12: *P. acnes* isolates showing high OD indication more production of biofilm. Row A4-A9, D1-D3, F7-F9: *P. acnes* isolates showing moderate OD indication more production of biofilm. Row G : *P. acnes* ATCC 11828; Row H : Medium control; Remaining wells: *P. acnes* isolates showing absence of biofilm production.

for typing of *P. acnes*. The predominant RFLP pattern was type IB followed by type IC. Type IA was seen only in conjunctival swabs. Type II RFLP patterns were seen in preoperative swabs, traumatic keratitis and endophthalmitis.

An association of *P. acnes* with low pathogenicity and very delayed onset of pseudophakic endophthalmitis has been reported[23], and biofilm like deposits have been seen on IOLs with polymerase chain reaction confirmation[23]. Contact between lens and external tissues surrounding incision has been observed to result in 26 per cent of the lenses becoming colonized[23]. IOL appears to provide a niche where bacteria may attach to form a biofilm.

In our study the rate of biofilm formations was 16 per cent among isolates of *P. acnes* and majority was from conjunctival swabs. Biofilm producing *P. acnes* isolates showed resistance to more than three antimicrobial agents compared with those lacking this ability. Thus, existence of biofilm production among *P. acnes* isolates may suggest an increased threat of emergence and spread of antimicrobial resistance among *P. acnes*.

Direct intravitreal instillation of antibiotics to treat endophthalmitis, caused by many anaerobic organisms has been shown to be safe and effective[34]. In the present study, all *P. acnes* ophthalmic isolates demonstrated high-level *in vitro* susceptibilities to ceftazidime as also reported in our previous study[32]. All patients were treated with topical ciprofloxacin and moxifloxacin eye drops and intravitreal injection of ceftazidime, cefazolin, amikacin and vancomycin but vancomycin resistance was seen in our previous study[32]. Increasing vancomycin resistance needs to be checked in future studies. Amphotericin B, natamycin and fluconozole were used as antifungal agents for the treatment of fungal co-infection with *P. acnes*.

In conclusion, our study findings demonstrated the various subtypes of *P. acnes* by targeting *16S rRNA* among ocular isolates by PCR-RFLP technique, which was comparatively more cost-effective than the DNA sequencing method and achieved rapid differentiation of subtypes of *P. acnes*. No association was observed between *P. acnes* subtypes and presence/absence of biofilm. Further investigations are required to explore the relationship with different subtypes of *P. acnes*, their inflammatory response, their interaction with the ocular cellular mechanism and their role in exhibiting both virulence and pathogenicity. Such studies will pave the way for better understanding of its pathogenicity, virulence mechanism and efficient antibiotic treatment along with the clinical management of ocular *P. acnes* infections.

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***Conflicts of Interest*:** None.

Table III. Minimum inhibitory concentrations (MICs) of high and moderate biofilm producing *P. acnes* isolates (n=16)

S. No.	Specimen	Ciprofloxacin		Norfloxacin		Cephotaxime		Metronidazole		Imipenum		Vancomycin		Clindamycin		Nalidixic acid	
		MIC = (**µg/ml??**) and susceptibility status															
		High biofilm producing isolates															
1	Conjunctival swab	0.2	S	0.5	S	2	S	2	R	1	R	2	R	2	R	4	R
2	Conjunctival swab	0.4	S	4	R	1	S	2	R	2	R	2	R	2	R	4	R
3	Conjunctival swab	0.1	S	0.1	S	1	S	2	R	2	R	2	R	2	R	4	R
4	Conjunctival swab	0.1	S	2	R	1	S	2	R	2	R	2	R	2	R	4	R
5	Conjunctival swab	0.5	S	0.5	S	0.1	S	2	R	2	R	2	R	2	R	1	S
6	Conjunctival swab	0.4	S	2	R	0.2	S	2	R	0.1	S	2	R	2	R	4	R
7	Conjunctival swab	0.1	S	2	R	1	S	1	R	2	R	1	S	2	R	4	R
8	Eviscerated material	0.1	S	2	R	0.2	S	2	R	2	R	0.5	S	2	R	4	R
9	Eviscerated material	0.1	S	2	R	0.2	S	2	R	2	R	1	S	0.5	S	4	R
10	Eviscerated material	0.1	S	2	R	0.2	S	2	R	2	R	1	S	1	S	4	R
11	Eviscerated material	0.2	S	2	R	0.2	S	2	R	2	R	2	R	2	S	4	R
12	Corneal scrapping	0.2	S	4	R	1	S	1	R	2	R	4	R	0.2	S	4	R
		Moderate biofilm producing isolates															
1	Conjunctival swab	0.4	S	4	R	0.1	S	0.1	S	2	R	0.2	S	2	R	4	R
2	Conjunctival swab	0.2	S	2	R	1	S	1	R	2	R	2	R	0.2	S	2	S
3	Conjunctival swab	0.4	S	4	R	2	S	1	R	2	R	2	R	0.2	S	2	S
4	Eviscerated material	0.2	S	2	S	0.2	S	0.1	S	2	R	2	R	0.1	S	4	R

R, resistant; S, susceptible

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