Ocular Propionibacterium Acnes: A Study On Antibiotic Susceptibility Profiling And Their Epidemiological Pattern

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Citation

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Abstract

Purpose: To identify the antibiotic pattern of commonly used P. acnes isolates and to analyse the epidemiological patterns using Random Amplification of Polymorphic DNA (RAPD) technique. Methods: One hundred P. acnes isolates (90 % extraocular and 10% intraocular) recovered from ocular clinical specimens identified by conventional method were studied. MIC of ciprofloxacin, norfloxacin, nalidixic acid, clindamycin, penicillin G, vancomycin and metronidazole, cefotaxime and imepenum were carried out for 100 P. acnes ophthalmic isolates by spot inoculation technique. Results: Six distinct RAPD patterns were observed among these isolates of which the 4th pattern was the most predominant one (41%), which was isolated mostly from conjunctival swabs. Of the 100 clinical anaerobic P. acnes isolates, highest resistant antibiotic pattern was seen among conjunctival swabs isolates for metronidazole (100%). Following it was the clindamycin (79%) and penicillin G (64%). Conclusion: This study has proven that RAPD is a reproducible, powerful technique for P. acnes genomic typing and may definitely play a vital role in identifying epidemiology of P. acnes. There was no correlation between the antibiotic pattern and the corresponding RAPD fingerprinting results in our study. Resistance to Vancomycin, the most commonly used intravitreal antibiotic is emerging among P. acnes is being reported for the first time. Studies at molecular level with vancomycin resistant isolates will help to understand the mechanism of resistance.

INTRODUCTION

Propionibacterium acnes is a major inhabitant of the adult human skin and conjuctival sac.[1, 2] Currently, routine diagnostic practices underestimate the clinical importance of this anaerobic organism due to inefficient detection and isolation procedures, along with the traditionally held view that, the organism has low virulence; its presence in clinical samples reflects contamination with normal flora.[3] However the organism is gaining clinical importance. P. acnes is responsible for post surgical delayed endophthalmitis[4], lacrimal sac and/or nasolacrimal duct obstruction[5], infections of prosthetic implants[6], intraocular lenses (IOLs)[7], keratitis.[8]

It is not known whether P. acnes that reside as normal flora and that isolated from disease sites have any genetic variations. Very few studies have been published on the epidemiology of P. acnes isolates. Random amplification of polymorphic DNA (RAPD) technique, a modification of Polymerase Chain Reaction (PCR), in which a single primer is able to anneal and prime at multiple locations in a genome, can be of great epidemiological value.[9] The success of this method is due to the fact that no prior information about the target sequence is needed. These primers detect polymorphisms in the absence of variations and may work as genetic markers that are used in epidemiologic studies.

Increasing prevalence of antimicrobial resistance to P. acnes has been documented since last 20 years.[10] To treat such infections adequately, empirical therapy usually relies on the use of Beta-lactams (penicillin's, third-generation cephalosporins) combined with metronidazole and/or vancomycin. Depending on the circumstances, other antibiotics such as clindamycin or fluoroquinolones may also be used.[11]

P. acnes is the most predominant anaerobe isolated from ocular specimens in our hospital set up. Since there are very few reports for understanding the epidemiological pattern of P. acnes available in literature and their correlation with the antibiotic pattern of P. acnes, the current study was undertaken. This is the first study carried out in India with the P. acnes isolates recovered from ocular specimens.

MATERIALS AND METHODS

BACTERIAL STRAINS

One hundred P. acnes isolates recovered from ocular clinical specimens (conjunctival swab - 65, conjunctival scraping – 1, corneal scraping – 13, lid abscess – 3, iris tissue - 2, corneal button-5, vitreous chamber tap – 1, anterior chamber tap - 2, eviscerated material – 6, orbital implant – 1 and sclera nodule - 1) received at L & T Microbiology Research centre, Sankara Nethralaya, a tertiary eye care centre at Chennai, India along with 3 American Type Culture Collection (ATCC; Manassas, Va.) (Propionibacterium acnes ATCC 11828, Clostridium sporogenes ATCC 11437 and Bacteroides fragilis ATCC 23745) were included for this study. The institutional research sub-committee approved this study protocol. Clinical specimens were processed as described elsewhere.[12]

BACTERIAL CULTURE

Ocular anaerobic culture isolates received from individual patients proven to be P. acnes by conventional methods including Gram staining, catalase, indole positivity, growth characteristics in Brucella blood agar enriched with 5% sheep blood, 5 mg/L haemin and 1 mg/L vitamin K1, (Cat No. M 1039, Hi-Media Laboratories Private Limited, Mumbai) were studied. The isolates were maintained at anaerobic work station (Don Whitley Scientific Limited, Shipley, West Yorkshire, UK) (85% N_2, 10% H_2, 5% CO_2) through out the study period. The growth on Brucella blood agar was observed to be small, round, yellow to orange colored shiny and convex spherical beta haemolytic colonies.

OPTIMIZATION OF RAPD TECHNIQUE

To optimize the RAPD fingerprinting technique, the optimal concentrations of primers, DNA template, and $MgCl_2$ used in PCR were first determined. Trying different concentrations ranging from 15, 30, 60 and 100ng/µl the primer concentration was subjected for optimization. Primer dimer formation was seen with the concentrations of 60 and 100ng/µl. With 15 ng/µl primer concentration the bands were very faint. Primer concentration of 30ng/µl yielded clear banding patterns. Concentration of $Mgcl_2$ ranging from 1 to 4mM $MgCl_2$ produced identical banding pattern, higher concentrations of $MgCl_2$ yielded some artificial background, and lower concentrations of $MgCl_2$ resulted in poor amplification. A concentration of 2mM $MgCl_2$ was chosen for the RAPD reaction. Likewise, low levels of DNA template (5, 10 ng) were found to result in relatively poor amplification while higher level yielded smearing patterns. RAPD patterns, however, standardized with 20ng/ul of total DNA were used. Consequently, a slight difference in DNA concentration obtained from preparation to preparation did not affect RAPD patterns. [13, 14]

To select suitable RAPD primers for subtyping of 100 P. acnes isolates, 10 arbitrary primers were first tested with 10 isolates of P. acnes and one primer , 7 th primer (Accession no for 7 th primer is AM773318, Merck, Darmstadt, Germany) was found to produce three or more polymorphic patterns when tested, was taken for the RAPD determination. [15, 16]

PCR-RAPD PROCEDURE

DNA was extracted from a single colony isolated from Brucella blood agar using the Qiagen DNA Mini kit (Qiagen, Hilden, Germany) as per the instructions provided in the manual. The resulting genomic DNA was stored at -20°C until further subjected for PCR-RAPD analysis. PCR-RAPD was carried out for all P. acnes isolates by using 20 ng/µl of genomic DNA added to 20µl of PCR mixture consisting of 5µl 10X PCR buffer containing 15mM $MgCl_2$, 200µM dNTPs, 100ng / µl of primer, 10µl deionized water of and 1.0 U of Taq polymerase (Merck, Darmstadt, Germany). PCR amplifications were carried out using thermal cycler Perkin Elmer Model 2700 (Applied Biosystems, Massachusetts, USA). PCR thermal profile consisted of initial denaturation at 94°C for 5 min, followed by 8 cycles consisting of denaturation at 94°C for 45sec, annealing at 35°C for 1 min, and extension at 72°C for 1.5 min, followed by 35 cycles consisting of denaturation at 94°C for 45sec, annealing at 38°C for 1 min, and extension at 72°C for 1 min and a final extension at 72°C for 10 min.

Entire RAPD products were loaded into 1% agarose gel (SRL, India) containing 1x Tris-acetate-EDTA buffer. Molecular size markers (100bp and 500 bp DNA ladder; (Merck, Darmstadt, Germany)) were run in parallel on all gels. Resolved DNA products were stained with ethidium bromide, 50ng/ml (Hi-Media Laboratories Private Limited, Mumbai.) and viewed under UV light using gel documentation system (VILBER LOURMAT – FRANCE). [15, 16]

ANTIBIOTIC SUSCEPTIBILITY TESTING

The following antibiotics powders were used and obtained from the sources indicated: ciprofloxacin, norfloxacin, nalidixic acid, clindamycin, penicillin G, vancomycin and metronidazole (Sigma, USA), cefotaxime and imepenum

(Ranbaxy, India) were used to make stock solutions that were filter sterilized and then stored at 4ºC. Final working solutions tested for ciprofloxacin, norfloxacin, nalidixic acid and vancomycin were of 0.125, 0.25, 0.5, 1, 2 and 4 mg/L respectively. For clindamycin, penicillin G, cefotaxime and imipenum, the final concentrations tested were 0.06, 0.125, 0.25, 0.5, 1 and 2 mg/L respectively. Metronidazole final concentrations were 8, 16, 32, 64, 128 and 256 mg/L respectively. [17 - 21]

DETERMINING OF MINIMUM INHIBITORY CONCENTRATION (MIC)

MICs of nine antimicrobial agents were determined by agar dilution technique as described by the Clinical Laboratory Standard Institute (CLSI) standard with 10^5 CFU spot and Brucella base sheep blood agar.[20, 21] The plates were incubated in Don Whitley anaerobic work station (Shipley, West Yorkshire, UK) for 48 h at 37°C. The MIC was defined as the lowest concentration of antimicrobial agent that resulted in a marked change in the appearance of growth in comparison with the control plate, as described in the NCCLS protocol. P. acnes ATCC 11828 was used as control strain.

RESULTS

In the present study, a total of 100 P. acnes isolates from various ocular specimens were included. The highest rate of P. acnes isolation (65%) was from the conjunctival swabs.

PCR-RAPD PATTERN

PCR-RAPD analysis performed with 100 P. acnes isolates with the 7 th set of RAPD primers showed 6 different RAPD patterns distributed among P. acnes positive ocular isolates is shown in and figure 1.

Pattern 1 was exhibited by 3 (3%) isolates of P. acnes [conjunctival swabs - 2 and eviscerated material – 1]. The products had 6 bands measuring 2500, 1500, 900, 800, 400, 100bp in size. Pattern 2 was exhibited by 18 (18%) isolates [anterior chamber tap – 1, conjunctival scraping – 1, conjunctival swab – 14, iris tissue – 1 and corneal scraping – 1]. There were totally 7 Bands with size measuring 2000, 1200, 900, 500, 400, 300, 100 base pairs. Pattern 3 was exhibited by 16 (16%) isolates [conjunctival swab - 12, corneal button – 1, corneal scraping – 2 and eviscerated material - 1]. The products produced 7 bands measuring 2000, 1200, 900, 600, 500, 400, 100bp in size. Pattern 4 was shown by 41 (41%) isolates [anterior chamber tap - 1, conjunctival swab - 22, corneal button - 4, corneal scraping – 7, eviscerated material – 2, iris tissue – 1, lid abscess – 3, sclera nodule - 1], with 5 products of size 2000, 1200, 900, 300, 100bp respectively. Pattern 5 was exhibited by 14 (14%) isolates. [Conjunctival swab - 11, corneal scraping – 2, lid abscess – 3, vitreous chamber tap – 1] There were totally 7 Bands measuring 1200, 1000, 800, 600, 500, 400, 100bp in size. Pattern 6 was shown by 8 (8%) isolates [conjunctival swab - 4, corneal scraping – 1, eviscerated material – 2 and orbital implant – 1] with 7 bands corresponding to 3000, 1500, 800, 600, 500, 300, 100bp in size respectively.

The 4 th pattern was found to be the most prevalent pattern among P.acnes. These different patterns from different specimens indicate that all are not of common origin or hospital acquired infection.

REPRODUCIBILITY OF RAPD FINGERPRINTING

The reproducibility of the RAPD fingerprinting technique was confirmed by comparing the reproducibility of the fingerprint patterns obtained from duplicate runs of RAPD analysis of several different P. acnes isolates. A single primer (7 th primer) was used to discriminate P. acnes species based on their sequence variation. The experiments were carried out twice using two different brands of thermal cyclers, and the results were resolved on the same 1% agarose gel to evaluate the reproducibility.

DETERMINATION OF MICS

Antibiotic susceptibility pattern and MIC values of 100 P. acnes isolates for the 9 antibiotics tested are shown in Table 1. The MICs for control strains were always within recommended limits. Figure 2 represents the graph showing the results of antibiotic resistance pattern prevalent among ocular P. acnes.

NORFLOXACIN

Of the clinical isolates, 41 isolates (41.0%) were resistant to Norfloxacin. The most resistant isolates were associated with conjunctival swabs (80%) followed by eviscerated material (80%) and corneal scraping (80%). The MICs for isolates ranged between <0.1 to 4mg/L.

PENICILLIN G

Of the clinical isolates, 64 isolates (64.0%) were resistant to penicillin G. The most resistant isolates were associated with conjunctival swabs. The degrees of resistant isolates were high with conjunctival swabs (40%) followed by corneal scrapping (33%) and eviscerated material (75%). The MICs

for isolates ranged between <0.1 to 2mg/L.

IMIPENUM

A total of 61 (61.0%) clinical isolates were resistant to imipenum. The most resistant isolates were associated with conjunctival swabs (42%) with the MICs ranging between <0.1 to 2mg/L.

VANCOMYCIN

Of the clinical isolates, 50 isolates (50.0%) were resistant to vancomycin. The most resistant isolates were associated with conjunctival swabs followed by eviscerated material and corneal scrapping with MICs ranging from between <0.1 to 4mg/L.

NALIDIXIC ACID

Of the clinical isolates, 42 isolates (42.0%) were resistant to nalidixic acid. The most resistant isolates were associated with conjunctival swabs. The MICs were ranging between <0.1 to 4mg/L.

CLINDAMYCIN

A total of 79 (79.0%) clinical isolates were resistant to clindamycin. Resistance to clindamycin was found to be predominant among conjunctival swabs isolates with MICs ranging from between <0.1 to 2mg/L.

CIPROFLOXACIN

All the isolates were susceptible to ciprofloxacin.

CEPHOTAXIME

All the isolates were susceptible to cephotaxime.

METRONIDAZOLE

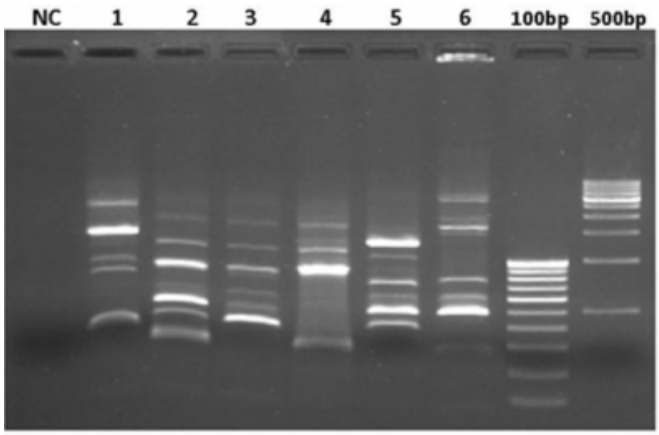
All P. acnes strains (100%) were resistant to metronidazole with an MIC >256 mg/L.

CORRELATION OF RAPD PATTERN WITH THE MIC RESULTS

There existed no correlation between specific banding patterns of RAPD with that of the MIC results. Higher resistance was seen among the extra ocular isolates especially the conjunctival swabs and however predominantly many conjunctival swabs exhibited pattern 4.

Figure 1

Figure 1: Agarose Gel electrophoretogram of six different RAPD finger printing profiles of the using 7 primer set.



Pattern 1 - 6 bands measuring 2500, 1500, 900, 800, 400, 100bp in size.

Pattern 2 - 7 Bands measuring 2000, 1200, 900, 500, 400, 300, 100bp in size.

Pattern 3 –7 Bands measuring 2000, 1200, 900, 600, 500, 400, 100bp in size

Pattern 4 – 5 Bands measuring 2000, 1200, 900, 300, 100bp in size

Pattern 5 – 7 Bands measuring 1200, 1000, 800, 600, 500, 400, 100bp in size

Pattern 6 - 7 Bands measuring 3000, 1500, 800, 600, 500, 300, 100bp in size

Figure 2

Figure 2: Graph showing the results of antibiotic resistance pattern prevalent among ocular

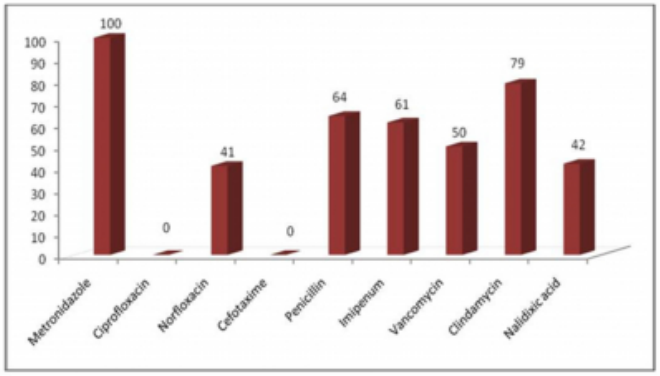


Figure 3

Table 1: Results of In-vitro susceptibility of 100 isolates

Antibiotics	Susceptible MIC for range (mg/L)	MIC for isolates (mg/L)	No of isolates resistant
Ciprofloxacin	≤ 0.25 to 2 mg/L	≤ 0.1 to 4mg/L	0 %
Norfloxacin	≤ 0.12 to 1 mg/L	≤ 0.1 to 4mg/L	41 (41.0%)
Nalidixic acid	≤ 0.25 to 2 mg/L	≤ 0.1 to 4mg/L	42 (42.0%)
Clindamycin	≤ 0.06 to 2 mg/L	≤ 0.1 to 2mg/L	79 (79.0%)
Vancomycin	≤ 0.5 to 2 mg/L	≤ 0.1 to 4mg/L	50 (50.0%)
Cefotaxime	≤ 0.06 to 1 mg/L	≤ 0.06 to 2mg/L	0 %
Imipenum	≤ 0.06 to 1 mg/L	≤ 0.1 to 2mg/L	61 (61.0%)
Metronidazole	≤ 0.06 to 1 mg/L	≥256 mg/L	100 (100.0%)
Penicillin G	≤ 8 to 256 mg/L	≤ 0.1 to 2mg/L	64 (64.0%)

Performed against nine antimicrobial agents

DISCUSSION

P. acnes is the msot predominant anaerobe isolated from the conjunctival specimens. [22, 23]The P. acnes endophthalmitis is increasingly being reported in cases of delayed type post surgical endophthalmitis. Over all isolation rate of P. acnes from patients was found to be 4.2 % per year approximately and predominantly from conjunctival swabs specimens. Importance was given to these conjunctival swabs isolates because P. acnes survive as the normal conjunctival flora of the conjunctiva and may gain access into the eye during surgery and cause post-operative endophthalmitis. A detailed study performed with a large collection of ocular isolates of P. acnes is rarely reported in literature. In the current study, we analyzed 100 isolates of P. acnes recovered from various ocular specimens.

Of the 100 P. acnes isolates included in our study 90% of them were from extraocular specimens and 10% were from intraocular specimens. Predominant extra ocular specimen was conjunctival swabs belonging to patients reporting in our hospital to undergo any form of eye surgery. The remaining extraocular isolates belonged to patients with a previous history of injury or trauma or an insect bite. The anaerobic infection in them was considered to be of community origin but not nosocomial or hospital based. One of the main sources of conjunctival bacteria is the adjacent skin flora. Since Propionibacterium is the predominant anaerobic organism of the skin, it is reported to play the same role in the conjunctiva as well. [24]

PCR- RAPD fingerprinting technique is a particularly powerful taxonomical tool. It is proven that the microheterogenecity of the sequences among strains arises due to continuous point mutations and other variations in the genome, which results in various RAPD patterns. This method utilizes arbitrary oligonucleotides to prime DNA synthesis at low annealing temperatures to divulge genomic diversity. Therefore this powerful tool was applied to study the heterogenecity among P. acnes isolates. [25]

Studies reported earlier have shown the existence of only two genotypes of P. acnes. [3] In our study we found a total of 6 RAPD patterns, of which pattern four was predominantly present among P. acnes isolates obtained from various ocular specimens. There existed no specific pattern for any particular type of specimen. Our study also showed that PCR-RAPD could be applied for large-scale genotyping of isolates of P. acnes.

As reported earlier by Pilu Dali et al., [26] all the P. acnes ophthalmic isolate from our study demonstrated excellent high-level in vitro susceptibilities to ciprofloxacin and cephalosporins based on the MIC data. Nayak N et al., has reported that the world literature review highlights the strains of P. acnes showing resistance not only to ciprofloxacin, but also to fourth generation fluoroquinolones like gatifloxacin and moxifloxacin. Whereas in our study we found that isolates were resistant to norfloxacin but 100 % susceptibility was seen for ciprofloxacin.[4] Almost all the patients with P. acnes positivity were treated with ciprofloxacin eye drops and significant clinical response has been observed.

All P. acnes strains (100%) were resistant to metronidazole >256 mg/L as reported by many in literature (2, 26).

In addition to this, ophthalmic isolates of P. acnes was proven to be resistant to imepenum and vancomycin with the MIC values ranging between 0.1mg/ml to 2.0mg/ml and 0.1mg/ml to 4mg/ml respectively.

In a study conducted by Mory et al. (11) and Oprica et al. [18] on antibiotic susceptibility pattern of P. acnes, they found 100% susceptibility to vancomycin and penicillin.[18] All these antibiotics play a vital role in treating patients with clinically suspected P. acnes endophthalmitis. In our study group, 50 % of isolates showed resistance to vancomycin with an MIC of < 0.1 to 4mg/L and 64 % isolates showed resistance to penicillin with an MIC of < 0.1 to 2mg/L.

Miriam A. Smith et al. [20] Mory et al. [21] found that all P. acnes strains that were tested were highly susceptible to cefotaxime, imipenem and clindamycin. Our isolates showed 100 % susceptibility to cefotaxime. Resistance to clindamycin was about 79 % followed by 61% resitance to

imepenum. This indicates the emergence of resistance to clindamycin and imepenum among ocular isolates.

To conclude, this study has proven that RAPD is an alternative rapid, reproducible, powerful genomic typing method for P. acnes and may definitely play a vital role in identifying epidemiology of P. acnes. In our study we could not establish any correlation between the antibiotic resistance pattern of the isolates and RAPD patterns. This is the first study to report on antimicrobial susceptibility by MIC method for 9 antibiotics along with the demonstration of PCR-RAPD on P. acnes with reproducibility of results and screening of correlation between both MIC results and RAPD pattern from a large number of ocular isolates of P. acnes in the literature. Vancomycin resistance among the P. acnes is being reported for the first time. Studies at molecular level with vancomycin resistant isolates will help to understand the mechanism of resistance better.

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