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Post-operative endophthalmitis: Antibiogram & genetic relatedness between *Pseudomonas aeruginosa* isolates from patients & phacoemulsifiers

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***Background & objectives*: Though not frequently but there are reports showing phacoemulsifiers as a potent source of infection in post-operative cases of endophthalmitis. This study was carried out to find antibiogram and genetic relatedness between *Pseudomonas aeruginosa* isolates from a post-cataract surgery endophthalmitis outbreak (3 patients) and internal tubings of 5 phacoemulsifiers.**

***Methods*: *In vitro* antimicrobial sensitivity patterns of the 8 bacterial isolates were observed. Genetic analysis of the bacterial isolates was done using random amplification of polymorphic DNA (RAPD) assay and PCR ribotyping. The resulting DNA band patterns were examined visually and by computer assisted analysis using unweighted pair group method.**

***Results*: The three *P. aeruginosa* patient isolates were found to be different from the five phacoemulsifier isolates in sensitivity towards 3 antibiotics and by genetic analysis (33 and 44% homology by RAPD assay and PCR ribotyping). Two of the patient isolates shared 100 per cent genetic homology by RAPD assay and another pair shared 100 per cent homology by PCR ribotyping. The five isolates from phacoemulsifiers did not share significant genetic homology. There was significant genetic variation between bacterial isolates from patients and phaco emulsifiers.**

***Interpretation & conclusion*: Though the three *P. aeruginosa* isolates obtained from the patients were phenotypically similar and genetically close, they differed from the phaco-machine isolates both genetically, and in their antibiogram profile. However, the five phacoemulsifier isolates were genetically diverse though they shared the same antibiogram profile. Therefore the Ringer's lactate from phacomachines could not be conclusively proven to be the source of infection.**

Key words Antimicrobial sensitivity pattern - PCR ribotyping - phacoemulsifiers - post-operative endophthalmitis - *Pseudomonas aeruginosa* - RAPD assay - Ringer's lactate

Post-operative endophthalmitis after cataract surgery, though rare, is still being reported[1]. It is one of the major devastating complications of intra-ocular surgery[1,2]. Gram negative organisms are implicated less frequently as causative agents compared to Gram-positive organisms[2,3]. A majority of the episodes of

endophthalmitis caused by Gram-negative bacteria are due to *Pseudomonas aeruginosa* and members of *Enterobacteriaceae*[3].

Although bacteria derived from the patient's own commensals and colonizing flora[4-6], are responsible for most outbreaks; exogenous sources such as operating team, operating room air, contaminated intraocular lenses, irrigation fluids and surgical equipment may also contribute to a certain extent[4,7-9]. There are scanty reports of post-operative cases of endophthalmitis in which phacoemulsifiers were the potent sources of infection[10,11] Regardless of the source, it often becomes obligatory on the part of the microbiologist and the ophthalmologist to strain type the outbreak isolates in order to determine the source, especially so in a tertiary care setting.

Though antibiotic sensitivity patterns are good indicators of typing, molecular methods like randomly amplified polymorphic DNA (RAPD) assay and polymerase chain reaction (PCR) ribotyping have extensively been used in the molecular epidemiology of different outbreaks and episodes of infections[12-14].

Here we report a cluster of cases of *P. aeruginosa* endophthalmitis following cataract surgery in which the same organisms were also isolated from the phacoemulsifiers. We investigated to find out antibiogram profile and genetic relatedness, if any, amongst the bacterial isolates form the patients and phacoemulsifiers.

Material & Methods

Bacterial isolates: A cluster of 3 endophthalmitis cases occurred after a single day's (August 2005) phacoemulsification cataract surgery on 98 patients at Dr Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India. All patients developed the features of endophthalmitis (symptoms of pain and redness and signs of cells and flare in anterior chamber, corneal oedema, poor glow and dull vision) within 24 h of surgery. Thus the vitreous specimens were collected from all the three subjects on the first post-operative day. At the same time, in order to identify the source of the episode, samples from all the probable sites in the patients' vicinity *i.e.* the instrument trays, dressing drum, chittel forceps, operating table, overhead light, surgeons' gloves, gowns and the phaco machines (Alcon Universal II, Texas, USA), were collected by rubbing 1cm^2 area on the surface with the help of a sterile cotton swab pre-moistened with sterile normal saline. The swabs were immediately streaked onto 90 mm diameter blood agar plates which were incubated at 37^{o}C. Ringer's lactate solution from the internal tubings of the phaco machines were inoculated onto thyoglycolate broth and incubated at 37^{o}C. Both the irrigation and the aspiration ports of the phacoemulsification probes were washed with sterile normal saline and the washings were inoculated onto the thyoglygolate broth for culture. Since there were 5 phaco machines within the operating room and all of them were in the running condition, we sampled all five. The person, who collected the samples from the machines, did so randomly without knowing, which three machines were used for the three patients. The organisms were identified and antibiotic sensitivity testing was performed according to the standard porotocol[15,16].

Antimicrobial susceptibility testing: The 8 isolates of *P. aeruginosa* were tested for their *in vitro* antimicrobial susceptibility patterns by employing the Kirby Bauer's disc diffusion method[16] by using antibiotic impregnated filter paper discs (Hi Media, Mumbai, India). The organisms were tested against (5 µg/disc) ciprofloxacin, tetracycline (30), Chloramphenicol (30), gentamicin (10), tobramycine (10), cephazolin (30), amikacin (30) and polymyxine B (300 units/disc). The results (susceptible/resistant) were interpreted by comparison of the zone size with the prescribed parameters according to the recommendations laid down by the CLSI (Clinical Laboratory Standard Institute) guidelines[17].

Genetic analysis of the bacterial isolates:

Genomic DNA isolation - Genomic DNA was isolated from pure log phase culture of *P. aeruginosa* by cetryl trimethyl ammonium bromide (CTAB)/NaCl method[18]. Breifly, the bacterial cell pellet from 5 ml log phase culture were resuspended in 567 µl TE buffer and lysed with 30 µl of 10 per cent of 20 mg/ml proteinase K (final concentration of 100 µg/ml proteinase K in 0.5% SDS) for 1h at 37^{o}C. It was mixed with 100 µl of 5M NaCl and 80 µl of CTAB/NaCl (Sigma, USA) (20% CTAB in 2.5M NaCl) and incubated at 65^{o}C for 30 min followed by phenol chloroform extraction and ethanol precipitation. The purified DNA was resuspended in TE, quantified and used in RAPD assay and PCR ribotyping. Reference ATCC strain of *P. aeruginosa* and one other laboratory isolate were included as controls in the genotyping assays.

RAPD assay - The PCR amplification for RAPD analysis was carried out using the primer M13 (5' GAGGGTGGCGGTTCT-3')[19]. The PCR assays were performed in 50µl volume containing 1.25U of *Taq* DNA polymerase (Invitrogen, USA) and 0.25µM each primer. The PCR assay was standardized with regard to $MgCl_2$ (2.5mM) and temperature profile (denaturation for 60 sec at 94°C, annealing for 150 sec at 30°C and extension for 150 sec at 73°C) and were carried out in thermal cycler (Gene Amp PCR System 9700, USA) for 35 cycles. Each experiment was carried out with the ATCC strain of *P. aeruginosa* and one unrelated laboratory isolate (LI) for reproducibility and accuracy of the experiment. The PCR amplified DNA was electrophoresed on 2 per cent agarose gel with the DNA molecular weight marker (100bp DNA ladder, GIBCO, BRL USA) and visualized after staining with (0.5µg/ml) ethidium bromide in a gel documentation system (SYNGENE, USA).

PCR ribotyping - PCR ribotyping was done by amplification of 16S-23S rRNA Intergenic Spacer Region (ISR) using universal primers forward (5' TTG TAC ACA CCG CCC GTC A 3') and reverse (5' GGT ACC TTA GAT GTT TCA GTT C 3')[14]. The PCR assay was standardized with 1.5mM $MgCl_2$ and 1U of *Taq* DNA polymerase (Invitrogen, USA). The temperature profile used was 60 sec at 95°C, 120 sec at 52°C, and 120 sec at 73°C. The amplification was done for 35 cycles in a thermal cycler (Gene Amp PCR System 9700, USA). The product was electrophoresed in 2 per cent agarose gel with the DNA molecular weight marker (100bp DNA ladder, GIBCO, BRL USA) and visualized after staining with (0.5µg/ml) ethidium bromide in a gel documentation system (SYNGENE, USA).

Computer-assisted analysis of the DNA banding patterns - The DNA band patterns obtained in RAPD assay and PCR ribotyping were analyzed by necked eye examination of the fingerprints and using the computer software SYNGENE (SYNGENE, Synotics Ltd., USA). Dendograms were constructed using the computer software. Computer assisted analysis; methods and algorithms used in this study were carried out or used according to the instructions of the manufacturers. The fingerprints were compared by the un-weighted pair group method (UPGMA) using arithmetic averages and the Dice similarity coefficient according to criteria suggested by Tenover *et al*[20]. A tolerance of 0.1 in band positions was applied during the comparison of the fingerprinting patterns. Identical DNA types were arbitrarily defined as those with homologies higher than 80 per cent. A genetic cluster was defined as the RAPD-type that was shared by two or more isolates. The discriminatory power was calculated by the formula of Simpson Index of diversity[20].

Results

P. aeruginosa was isolated from all the 3 patients and 5 phaco machines. None of the cultures from other probable sites yielded any positive results. The organisms could be isolated from only the internal tubings. The isolates obtained from patients were categorized as Group I [endophthalmitis patients (EOP) 1,2 and 3] and those from phaco machines as Group II [Alcon (A) II, III, IV, VI and VII] isolates.

Antimicrobial susceptibility testing: All the 8 isolates of *P. aeruginosa* were uniformly sensitive to ciprofloxacin and polymyxine B and resistant to tetracyclin, gentamicin and cephazoline. Whereas the Group I (patients) isolates were sensitive to tobramycin and amikacin, the Group II (phaco machine) isolates showed resistance to these two. In contrast, only Group II isolates were sensitive to chloramphenicol**.**

RAPD assay: The number of visible bands in the amplification pattern obtained in RAPD assay varied from 2 to 5. On the basis of visual examination, the three Group I isolates of *P. aeruginosa* differed markedly from the 5 group II isolates from the phaco machines. Two of the patient isolates (Group I : EOP1 and 2) and 2 phaco isolates (Group II: A II and A III) appeared to be very similar by visual examination of the band patterns (Fig. 1).

In computer analysis of the dendogram (Fig. 2) drawn on the basis of similarity matrix which was created using the Dice coefficient generated using pair wise comparison for each of the types, the isolates could be divided into 7 types. The cluster size consisted of one or two isolates each. The genetic similarity varied from 32 -100 per cent and the discriminatory index was 0.93.

Two of the patient isolates (EOP I & 2) were found to be genetically identical (100% similar) whereas the third one shared 69 per cent homology. The genetic similarity between the third patient isolate (EOP 3) and ATCC strain was 100 per cent.

Only 2 isolates recovered from phaco machines (A VI and A VII) were genetically identical (100% homology). The rest were genetically diverse and the variation was from 32-75 per cent (Fig. 2). The

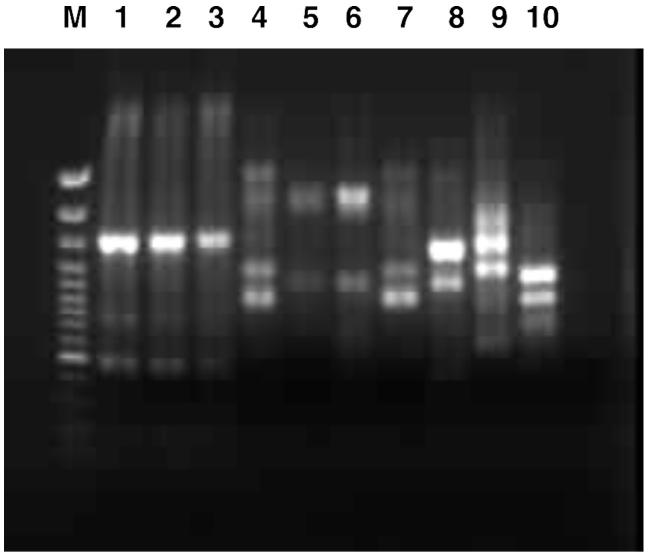

Fig. 1. Agarose gel showing DNA band patterns in RAPD assay. lane L, 100bp DNA LADDER; lane 1, EOP 1; lane 2, EOP 2; lane 3, EOP 3; lane 4, L I; lane 5, A II; lane 6, A III; lane 7, A IV; lane 8, AV I; lane 9, A VII; lane 10, Standard ATCC.

homology between Group I (patients) and Group II (machine) isolates varied from 32-66 per cent.

PCR ribotyping: The number of visible bands by PCR ribotyping varied from 1 to 3 and the Group I isolates were found to be markedly different from Group II isolates. All three Group I isolates produced identical banding patterns. In visual analysis, three of the Group II isolates (A II, A III and A VI) were similar in their band patterns (Fig. 3).

On the basis of similarity matrix created using the Dice coefficient and from pair-wise comparison for each of the genotypes and on the basis of dendogram drawn (Fig. 4), the isolates were divided into 4 PCR ribotypes. There were 1 to 4 isolates in each cluster. The genetic relatedness between the isolates of *P. aeruginosa* varied from 44 – 100 per cent and the discriminatory index was 0.91.

The similarity between the three patient isolates varied from 44-100 per cent even through two of the patient isolates (EOP 1 and EOP 3) showed 100 per cent homology. Moreover, these two isolates (EOP I and EOP 3) shared 100 per cent homology with two phaco machine isolates (A II and A III). The other patient isolate (EOP 2) shared 100 per cent homology with the ATCC strain of *P. aeruginosa* as well as with one of the machine isolates (A IV).

Only 2 isolates recovered from machines (A II & A III) were genetically identical (100% homology). The rest were genetically diverse and the variation was from 44-50 per cent (Fig. 4). The homology between Group I (patients) and Group II (machine) isolates varied from 44 to 100 per cent.

Discussion

P. aeruginosa is a leading pathogen amongst the Gram-negative bacteria causing endophthalmitis. *P. aeruginosa* endophthalmitis is a well described syndrome characterized by rapid progression and poor visual prognosis[21]. Although *P. aeruginosa* endophthalmitis can occur secondary to penetrating ocular trauma, perforating corneal ulcer or septicaemia, it is noted quite frequently following ocular surgery[3, 21].

Outbreaks of post-operative endophthalmitis due to *P. aeruginosa* were reported in the past, in which source of infection could be traced to the use of irrigating saline, intra-operative use of contaminated ophthalmic solutions or to the implantation of contaminated lenses[22-25]. However, there are only a few reports

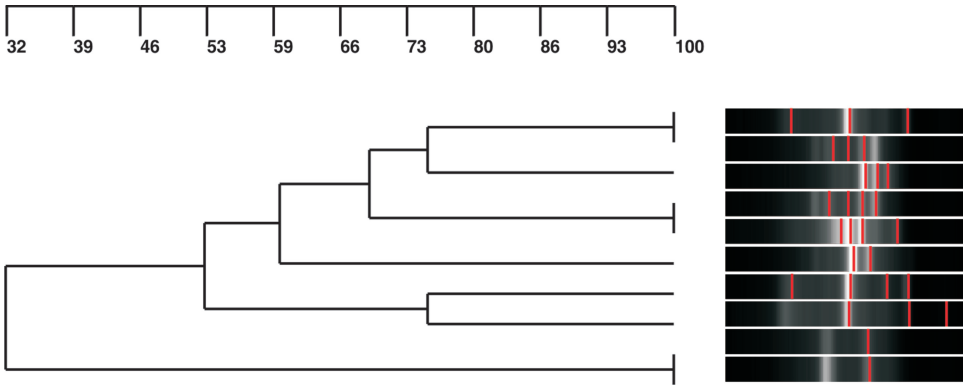

Fig. 2. Dendogram showing the homology on the basis of RAPD assay.

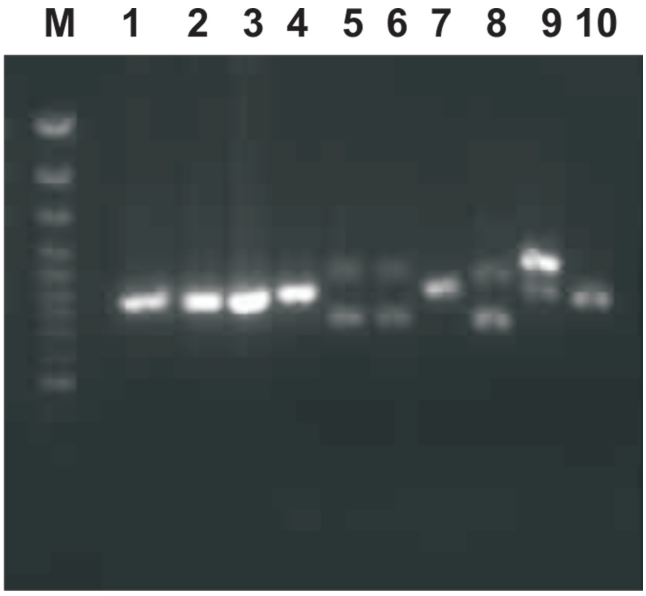

Fig. 3. Agarose gel showing DNA band patterns in PCR ribotyping. lane L, 100bp DNA LADDER; lane 1, EOP 1; lane 2, EOP 2; lane 3, EOP 3; lane 4, L I; lane 5, A II; lane 6, A III; lane 7, A IV; lane 8, A VI; lane 9, A VII; lane 10, Standard ATCC.

of post-operative endophthalmitis in which phaco-emulsifiers were the potent source of infection[10,11].

In the present study, isolation of *P. aeruginosa* isolates, from all the three patients having similar antibiotic susceptibility profiles could suggest a common source of infection. Of all the materials cultured, it was only the Ringer's lactate solution from the phacoemulsifiers' internal tubings which yielded the organisms. Even though retrograde flow from these is virtually impossible, microbiological monitoring of fluids from these is usually done to trace the source and to prevent phacoemulsifier associated infections.

We used two PCR based genotyping methods, in addition to the phenotypic method like determination of the antibiogram profile, in order to characterize and match the isolates obtained both from the patients and the phacoemulsifiers. The two PCR based methods were chosen because of ease of performance and the ability to provide quick and reliable results[12-14]. We observed, the discriminatory power of the RAPD assay seemed to be superior to that of PCR ribotyping method. Cruciani and colleagues[26], using molecular tool like pulsed field gel electrophoresis (PFGE) found that their isolates from the vitreous specimens from the cluster of three patients with endopthalmitis and those recovered from the internal tubing system of automated cataract surgical equipment had identical banding patterns of genomic DNA. In contrast, the three clinical isolates of *P. aeruginosa* in our study differed markedly both phenotypically and genotypically from those isolated from the internal tubings. In addition, all the three isolates, though were phenotypically identical by their antibiogram profile, showed some variations within them as evidenced by our genotypic characterization methods, that revealed 100 per cent homology between two isolates in the RAPD assay and similar homology amongst two others in the PCR ribotyping. This is in agreement with the observations made earlier on *P. aeruginosa* isolates using molecular tools like RAPD, PFGE and RFLP[12].

Malathi *et al*[27], by examining the DNA sequencing, found genetical identity amongst their *P. stutzeri* isolates from the AC taps and the irrigation port of the phacoemulsification probe. In another study, Zuluski and co investigators[11] documented that four cases of *P. aeruginosa* endophthalmitis occurred due to the contamination of the internal

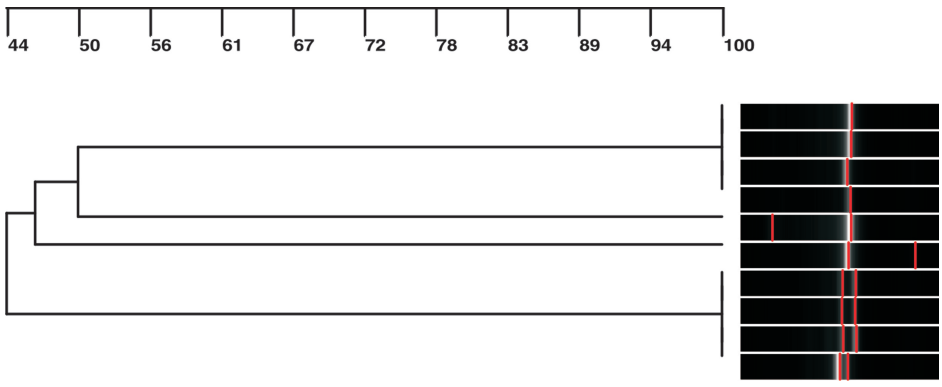

Fig. 4. Dendogram showing the homology on the basis of PCR ribotyping.

pathways of the pahcoemulsifier; because of the observed similar ribotyping patterns amongst the isolates from the vitreous samples and those from the phacoemulsifiers.

Our ribotyping analysis along with the RAPD assay results indicated that the *P. aeruginosa* isolates from the cluster of three patients operated on the same day were genetically close and had similar antibiogram pattern. Even though Ringer lactate solution from five phaco-emulsifier machines used on the same day yielded *P. aeruginosa* isolates with similar antibiotic sensitivity pattern these were genetically diverse and therefore Ringer lactate solution could not be conclusively proven to be the source of infection.

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***Conflict of interest*:** Nil

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