

Detection of β -lactamase Mediated Resistance among *Pseudomonas aeruginosa* Isolates from Ocular Infections

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ABSTRACT

Introduction: *P.aeruginosa* is the most prevalent gram-negative organism in ocular infections. Production of extended spectrum of β -lactamases (ESBL) by them may require alteration in the management protocol of gram-negative ocular infections.

Aim: To analyze the prevalence and detection of β -lactamase producing *P.aeruginosa* from ocular infections.

Materials and Methods: This study included all the patients with ocular infections who visited a tertiary care eye hospital in Coimbatore, for a period of four months. During this study period, the isolates were confirmed as *P.aeruginosa* by standard identification protocol. Minimum inhibitory concentration (MIC) of the antibiotics was determined by agar dilution method. Inducible β -lactamases and extended spectrum β -lactamases were detected by double disc diffusion method.

Results: Of the 2644 ocular specimens collected from 2618 patients, a total of 34 isolates of *P.aeruginosa* were obtained. MICs of cefotaxime and ceftazidime for all the isolates revealed that, 21 (62%) isolates were resistant to cefotaxime. A total of 11 strains were induced to produce β -lactamase with disc approximation at 20 mm apart and the presence of ESBL was detected in 4.3% of strains through 20 mm disc approximation, 34.7% of strains through 15 mm disc approximation. The synergic effect of cefotaxime with aminoglycosides was seen among 61.7% of strains.

Conclusion: Resistance of *P.aeruginosa* to β -lactam antibiotics seems to be prevalent in this part of the state. Combination of cefotaxime with aminoglycosides may be more useful for treatment of severe eye infections caused by *P. aeruginosa*.

Keywords: Antibiotics, Eye infections, Susceptibility

INTRODUCTION

Pseudomonas aeruginosa is a significant pathogen among gram-negative organisms infecting eye [1,2], because of its wide prevalence and resistance to antibiotics. In recent years, its resistance to β -lactam antibiotics and extended spectrum cephalosporins is of great concern to ophthalmologists worldwide. *Pseudomonas* resists β -lactam antibiotics by synthesizing β -lactamases [3]. These enzymes inactivate cephalosporins by hydrolyzing the amide bond of the β -lactam ring. The subsequent generation cephalosporins, which could overcome β -lactamases, are called extended spectrum cephalosporins, which include oxyimino β -lactams like ceftazidime and cefotaxime. Resistance to these antibiotics is by synthesis of extended spectrum β -lactamases (ESBLs), which are plasmid-mediated enzymes. These enzymes differ from their parent enzymes by only a few amino acid position but can hydrolyze extended spectrum cephalosporins [3].

Though these plasmid-mediated β -lactamases are observed in *P. aeruginosa* isolates in less than 2%, new classes of chromosomally encoded extended spectrum β -lactamases are also reported [3]. These enzymes have moderate hydrolysis activity for oxacillin but high activity against extended spectrum cephalosporins i.e., cefotaxime and ceftazidime.

The present study was therefore carried out in a tertiary care eye hospital in Coimbatore, south India to isolate *P. aeruginosa* from patients with ocular infections and to detect the synthesis of ESBL and their antimicrobial susceptibility pattern.

MATERIALS AND METHODS

The study was designed as a retrospective, laboratory based investigation which involved a total of 2644 (including repeat specimens) specimens (viz., corneal swab and corneal scraping) from 2618 patients processed from the patients attending Aravind Eye Hospital and Postgraduate Institute of Ophthalmology Coimbatore, India during January to April 2004. All the specimens that showed positive for bacteria in direct microscopy and culture media were included and specimens that grew fungal colonies were excluded from the study. The specimens were directly streaked onto solid media such as blood agar, chocolate agar, potato dextrose agar (PDA) and brain heart infusion broth and the plates/tubes were incubated at 37°C for 24 hours. The specimens were also placed onto clean, scratch free and labeled slide for gram staining. After incubation, the colonies were further subjected for gram staining and standard biochemical tests [4,5] so as to identify the causative organisms.

The isolates were stored as suspensions in a 10% (wt/vol) skimmed milk solution containing 10% (vol/vol) glycerol at -20°C until additional tests were performed. Only the confirmed *P. aeruginosa* strains isolated from ocular infections available from the storage facility (-20°C) were used in the study and none of the patient was directly involved.

Determination of minimum inhibitory concentration (MIC)

MIC of the antibiotics, cefotaxime and ceftazidime were determined by agar dilution technique [6,7]. Muller-Hinton agar containing cefotaxime and ceftazidime in final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 $\mu\text{g/ml}$ were used for the analysis. The MIC was defined as the lowest concentration of the drug inhibiting visible bacterial growth as evaluated after 18 hours incubation and the MIC breakpoints were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [6]. *P.aeruginosa* ATCC 27853 was used as a reference strain in every batch of MIC tests.

Detection of group I inducible β -lactamases

The prevalence of inducible β -lactamases was investigated by disc approximation method [7,8]. Cefotaxime (30 μg) disc was placed at distances 25 mm and 20 mm, respectively from a central cefoxitin (30 μg) disc on Muller-Hinton agar (MHA) plate swabbed with the lawn culture of the test isolate. After overnight incubation, distinct flattening of the inhibitory zone around the ceftazidime disc on the side nearest to the cefoxitin disc was regarded as an indication for the presence of inducible β -lactamase.

Detection of extended spectrum β -lactamase (ESBL) activity

The isolates were screened for the presence of ESBLs by the double-disc synergy assay [8]. Three ceftazidime (30 μg) discs were placed at distances 20, 15, and 10 mm, respectively, from a central amoxicillin-clavulanic acid disc on MHA swabbed with the lawn culture of the test isolate and the plates were incubated at 37°C overnight. The test result was considered positive when an enhancement of the inhibition zone around at least one of the ceftazidime disc toward the clavulanic-acid disc was observed [9].

Investigation of synergy effects of antibiotic combinations

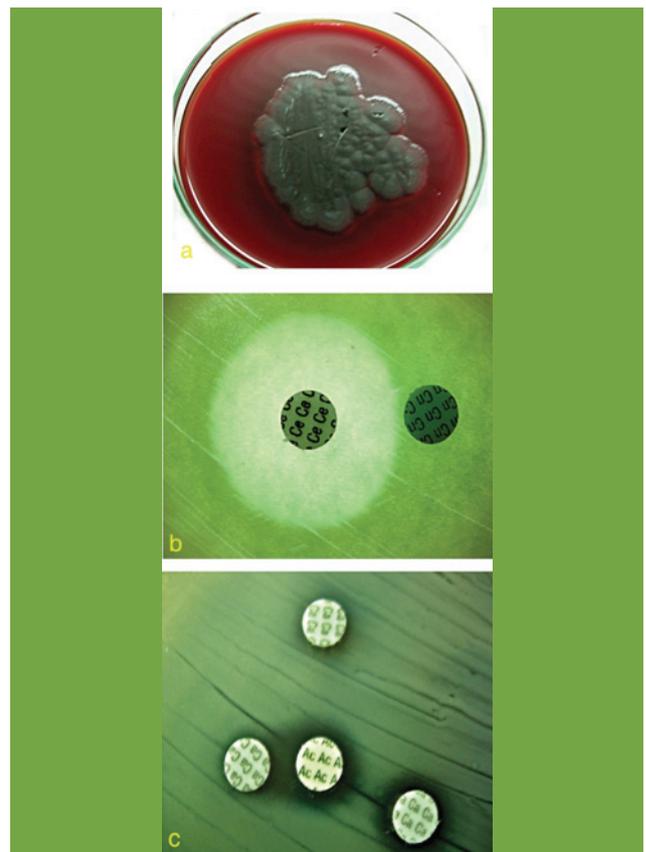
The synergy effects of the antibiotic combinations against the selected isolates were examined by disc diffusion test [8,10]. Two discs, each containing one or other of the two tested antibiotics (clavulanic acid and cefotaxime), were placed at a distance of about 20 mm from each other on MHA agar plate swabbed with the lawn culture of the test isolate. Synergy was considered to occur when there was a well-observed change (<2 mm) in the zone of inhibition. The synergy was classified as weak when a change <2 mm was observed in the zone of inhibition [10].

RESULTS

Most of the specimens 2079 (79%) were preoperative screening conjunctival swabs and only 108 (5%) specimens showed bacterial growth. Of these, *P. aeruginosa* grew in 3 (2.7%) cultures [Table/Fig-1a]. Only 565 (21%) specimens from 539 patients were processed for ocular infections.

P.aeruginosa grew in 34 cultures. Of these 21 (61.7%) isolates were cultured from corneal ulcer scrapings. MIC analysis revealed that, 21 (62%) isolates were found to be resistant to cefotaxime and 5 (15%) strains were found to be resistant to ceftazidime.

Isolates showing MIC value ≥ 16 mg/L were considered resistant for cefotaxime and ceftazidime (includes intermediate and resistant categories) [6]. In the present study 21 (61.7%) isolates showed resistance to cefotaxime. Out of these 5 (23.8%) isolates showed MIC of $\geq 64\text{mg/L}$ and 16 (76.1%) isolates had MIC ranges between 16-32 mg/L of cefotaxime. When MIC for ceftazidime was tested, a total of 5 (14.7%) isolates were completely resistant, 4 (88%) and 1 (20%) isolates had MIC $\geq 32\text{mg/L}$ and ≥ 16 mg/L, respectively.



[Table/Fig-1]: (a) Confluent growth of *P.aeruginosa* on blood agar (b) Detection of Inducible β -lactamase by double disk diffusion technique (The circumference of the inhibition zone around the cefotaxime disc is flattened on the side facing the cefoxitin disc, an inducible β -lactamase is inferred). (c) Detection of ESBLs by disc diffusion method at various disc distances. (The inhibition zone around the cephalosporin disc is extended towards the disc containing co-amoxiclav disc, considered as ESBLs producer).

Production of inducible β -lactamases was detected [Table/Fig-1b] among 11(32.3%) isolates, which were sensitive to cephalosporins with a zone of inhibition above 22 mm diameter. Extended spectrum β -lactamases were detected [Table/Fig-1c] in 23 (67.6%) isolates, which were resistant as per zone of inhibition. When the two discs (clavulanic acid and cefotaxime) were 25 mm apart, presence of ESBLs was detected in one (4.3%) isolate. When the distance was reduced to 15 mm, 8 (34.7%) isolates were found to produce ESBLs. At 10 mm disc approximation, 18 (78.2%) isolates were found

Various disc distances between Clavulanic acid and Cephotaxime	No. of positive isolates (%)	No. of negative isolates (%)
25 mm	1 (4.3)	22 (95.6)
20 mm	1 (4.3)	22 (95.6)
15 mm	8 (34.7)	15 (65.2)
10 mm	18 (78.2)	5 (21.7)

[Table/Fig-2]: Detection of extended spectrum β -lactamases by double-discsynergy method (resistant isolates n =23).



[Table/Fig-3]: Detection of invitro Drug synergy between Aminoglycosides and Cephalosporins by disc diffusion test. (Dumbbell shape of the two zones, considered as positive for in-vitro synergy).

Combination	No. of isolates showing synergy (%)	No. of isolates showing weak synergy (n%)	No. of isolates showing no synergy (n%)
Amikacin + Cephotaxime	15 (44.1)	6 (17.6)	13 (38.2)
Amikacin + Ceftazidime	13 (38.2)	6 (17.6)	15 (44.1)
Ofloxacin + Cephotaxime	17 (50)	4 (11.7)	13 (38.2)
Ofloxacin + Ceftazidime	13 (38.2)	7 (20.5)	14 (41.1)
Ciprofloxacin + Cephotaxime	17 (50)	4 (11.7)	13 (38.2)
Ciprofloxacin + Ceftazidime	13 (38.2)	7 (20.5)	14 (41.1)

[Table/Fig-4]: Synergy* effects of combinations of aminoglycosides and fluroquinolones with third generation cephalosporins. (Isolates n = 34).

* Synergy, well-characterized change of ≥ 2 mm in the inhibition zone. Weak synergy, change of < 2 mm in the inhibition zone. No synergy, no change observed in the inhibition zone (Mayer and Nagy, 1999).

Demographic Factors	Number of cases	Percentage (%)
Gender		
Male	18	53.0
Female	16	47.0
Age		
0-20	5	14.7
21-40	11	32.3
41-60	5	14.7
61-80	13	38.2
Occupation		
Agricultural	17	50.0
Construction	7	20.5
Students	3	8.8
Office	2	5.8
Unemployed	5	14.7
Specimen type		
Corneal scrapings	21	61.7
Contact lens	6	18
Vitreous	3	9
Conjunctival swab	3	9
Suture infiltrate	1	3
Total	34	100.0

[Table/Fig-5]: Profile of *Pseudomonas* infected cases taken for a study.

to produce ESBLs. By this method it was found out that 1 (4.3%) isolate produced only one type of ESBL and the other 18 (78.2%) isolates produced more than one type of ESBLs and 4 (17.3%) isolates did not produce ESBLs [Table/Fig-2]. Amikacin and ofloxacin when combined with cefotaxime exhibited a synergistic effect [Table/Fig-3] in 21(62%) on the isolates. When the combinations of amikacin and ofloxacin with ceftazidime were tested by this method, synergy was observed among 20 (58.8%) isolates [Table/Fig-4]

The epidemiological analysis of the patients revealed the following details. *Pseudomonas* sp. infections in eye did not show any sex predilection though the males were marginally more affected. Ocular infections by *P.aeruginosa* were seen among all age groups. The frequency of infection varied in different age group with more cases recorded in 61-80 age group [Table/Fig-5]. Most (50%) of the patients affected were agricultural workers followed by construction workers (20.5%).

DISCUSSION

Ocular infections cause profound morbidity and stand second to cataract in causing blindness worldwide [11]. Though trachoma leads among infective causes of blindness, the other ineffective causes are no less important in ocular infections, because of the morbidity they cause, and also because of

the increasing antibiotic resistance among bacterial isolates. It is becoming increasingly difficult to control ocular infections through antibiotics. ESBLs are now a problem in hospitalized patients throughout the world.

In this study, it was observed that 14.7% of strains were resistant to ceftazidime, and 61.7% of strains were resistant to cefotaxime. Bouza et al., [12] observed a similar finding where, 15% of the isolates of *P.aeruginosa* were resistant to ceftazidime. But Joan et al., [13] had reported that 53% of the *Pseudomonas* isolates tested were resistant to ceftazidime. Bonomo et al., [14] had reported that 46% of the *Pseudomonas* isolates were resistant to cefotaxime, where as in the study conducted by Joan [13], 93% of the strains were resistant to cephalosporins.

The induction of β -lactamases was seen in 36% of the isolates by using standard [7,8] approximation of 20 mm disc distance. This rate is low when compared to the earlier observation made by Moritz [15] who had demonstrated 68% of induction for cefotaxime using disc approximation test. But in the present study, all 100% strains were induced, after decreasing the disc distance between ceftazidime and cefotaxime by 5mm. This inducibility is significant because, different β -lactamases are induced at different disc approximations; hence by decreasing the disc distance, we may induce different types of β -lactamases, thereby proving the capacity of individual strains to induce these enzymes for exhibiting drug resistance [9].

A similar method was followed for detecting the presence of extended spectrum β -lactamases among resistance strains. By standard disc approximation method (25 mm) ESBL was detected in one isolate, but by reducing disc distances 78.2% of the isolates were able to produce ESBLs. There are more than 192 types of β -lactamases discovered [16]. These β -lactamases are produced at different concentration gradients of antibiotics [9]. Some of them are produced even at low level of selective pressure produced by these antibiotics and some require high antibiotic concentration for producing extended β -lactamases. By altering the disc distances, we can vary the concentrations and alter selective pressures of antibiotics to *P. aeruginosa*. This may be the possible reason for more isolates producing ESBLs at reduced disc approximations. According to Bert et al., [9], the standard test with 30 mm distance is insufficient to identify most ESBLs produced by *Pseudomonas* sp. Bradford et al., reported that, TEM derived enzymes and one PER-1 enzyme were detected by use of a 30 mm distance and it was necessary to reduce the distance to 20 mm to detect strains that produce VEB-1, SHV 29 and OXA 18. Whereas strains that produce, the other OXA derived ESBLs were identified only at a distance of 10 or 15 mm [9].

In this study, synergy between cephalosporins and aminoglycosides and cephalosporins and fluoroquinolones was detected by disc diffusion method based on Kirby-Bauer's antibiotic susceptibility testing as described by Mayer and Nagy [10]. The synergic effect of cefotaxime with aminoglycoside

was seen against 61.7% of strains and the synergic effect of cefotaxime with fluoroquinolones was also observed against 61.7% of strains. These combinations may be useful in treating the patients with *P.aeruginosa* but cephalosporin aminoglycoside combination is found to be more nephrotoxic than when drugs are used as mono therapeutic agents [17]. When combination of ceftazidime with aminoglycosides and fluoroquinolones were tested against *P.aeruginosa*, drug synergy was observed in 55.8% and 58.8% of isolates, respectively. Though in this study synergy was detected with disc diffusion test, measurement of time killing of the bacteria is the most reliable means of assessing the existence synergic effect between drugs [10]. The main mode of resistance among these organisms is through β -lactamase production and all strains can be induced to produce β -lactamases. *P. aeruginosa* can be agreed upon as the most dreaded gram-negative bacteria among ocular isolates and infections are very difficult to treat due to the ability of producing number of virulence factors and antibiotic resistance [18,19].

CONCLUSION

Our findings suggest that combinations of cefotaxime with aminoglycoside may be of significant value for the treatment of severe ocular infections caused by *P. aeruginosa*. Also, the alternative antibiotics such as imipenem may be considered for the unresponsive cases. Moreover, it's mandatory to detect β -lactamases in all the clinical laboratories, because these enzymes may significantly affect therapeutic outcomes.

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