ANTIMICROBIAL SUSCEPTIBILITY AND PREVALENCE OF EXTENDED SPECTRUM BETALACTAMASE (ESBL) AND METALLO BETALACTAMASE (MBL) AND ITS CO-EXISTENCE AMONG PSEUDOMONAS AERUGINOSA RECOVERED FROM OCULAR INFECTIONS

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Received: 11 Feb 2015 Revised and Accepted: 05 Mar 2015

ABSTRACT

Objectives: To evaluate the changing trends in antimicrobial susceptibility rate and detection of ESBL and MBL among Pseudomonas aeruginosa isolated from various ocular infections over a 2 year periods with special reference to detection of ESBL and MBL co-existence among P aeruginosa recovered from ocular infections.

Methods: All ocular specimens, culture positive for P aeruginosa (n=110) isolated from clinically suspected patients were submitted to L & T Microbiology Research Centre, Chennai, Tamil Nadu, India. Culture, antimicrobial susceptibility testing and ESBL detection was performed by Standard methods. MBL production was screened by Carbapenem-EDTA combination disk method.

Results: Of the 3247 samples subjected to culture from August 2012- July 2014 by standard method 276 were positive for bacterial growth, thereby 8.5% of ocular infections mediated by bacterial pathogens. Out of 276 culture positives 110 (39.89%) Pseudomonas aeruginosa isolates recovered from ocular infections. The resistance rate for commonly used drugs against ocular infection include Gentamicin [23.63%], Gatifloxacin [20.9%], Moxifloxacin [20%], Tobramycin [20%], ciprofloxacin [19.09%]. Totally 15 (13.63%) out of 110 isolates were identified as ESBL producer and 11 (10%) out of 110 isolates were identified as MBL producer by screening test, including 7 isolates have co-produced both ESBL and MBL enzymes and 4 isolates were only positive for MBL production.

Conclusion: Though fluoroquinolones remains a good choice for ocular Pseudomonal infection. Gradual emergence of resistance to fluoroquinolones and aminoglycosides also noted from this study. The emergence of ESBL, MBL and pandrug resistance among resistant mechanisms, because these enzymes are frequently transferable and inactivate beta lactam antibiotics for a broad range. Out of this large enzymatic family, carbapenemases, including carbapenems. The Class A extended spectrum beta-lactamases (ESBLs) hydrolyze penicillins, monobactams and cephalosporins whereas they are inhibited by the available beta-lactamase inhibitors [6].

Keywords: Antimicrobial resistance, Pseudomonas aeruginosa, Keratitis, Ocular infections.

INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) is a motile gram-negative rod that belongs to the family Pseudomonadaceae. P. aeruginosa, an ubiquitous organism frequently isolated from clinical specimens and accounts for a significant proportion of nosocomial infections [1]. A general problem with nosocomial infections is the tendency of the causative pathogen to acquire antibiotic resistance, whilst patient was under treatment [2]. P. aeruginosa is known for its ability to resist killing by a variety of antibiotics. The minimal nutritional requirements of Pseudomonas, as evidenced by its ability to grow in distilled water and its tolerance to a wide variety of physical conditions, contribute to its ecologic success and ultimately in its role as an effective opportunistic pathogen [3]. P aeruginosa is known for its ability to colonize the ocular surface even in the absence of clinical signs of infection. The mechanism of antibiotic resistance may be intrinsic (the microorganism is by definition resistant against a certain antibiotic) or acquired. Production of beta-lactamases is considered of major importance among resistant mechanisms, because these enzymes are frequently transferable and inactivate beta lactam antibiotics for a broad range. Out of this large enzymatic family, carbapenemases (class B) metallo beta-lactamases (MBLs) that contain zinc in their active site and class A KPC hydrolyze in vitro all or almost all beta-lactams, including carbapenems. The Class A extended spectrum beta-lactamases (ESBLs) hydrolyze penicillins, monobactams and cephalosporins whereas they are inhibited by the available beta-lactamase inhibitors [6].

Since, phenotypic method is an inexpensive, simple technique with fair reproducible result with easy interpretation method and can be used in any basic laboratories. The rational of this study is to detect and evaluate the ability of P aeruginosa recovered from ocular isolates for the production of ESBL and MBL enzyme neither single nor coproduction of both enzymes using phenotypic methods. In literatures, scanty reports on ESBL and MBL producers among isolates of P aeruginosa have been reported. In this context, the current study is a need for accurate and up to date information on the frequency and mode of resistance, resistance trends and comprehensive comparison of various antimicrobial agents tested against Pseudomonas aeruginosa in ocular infections.

Thus the information on the emergence of resistance with different antibiotics can be of practical use in guiding empiric therapeutic choices. In this study, we report the results of antibiotic susceptibility and multiple antibiotic resistance index of P aeruginosa isolates obtained from ocular specimens submitted to L & T Microbiology Research centre, Sankara Nethralaya, Chennai, Tamil Nadu, and India. The proposed study method can be used routinely to test antimicrobial sensitivity for accurate identification of resistant mechanism to guide the clinician for with appropriate drug regimen decision in the chemotherapy of P aeruginosa infections.

MATERIALS AND METHODS

A prospective analysis was done with 110 Pseudomonas aeruginosa isolated from patients with clinical diagnosis of bacterial ocular
diseases, namely conjunctivitis, canaliculitis, keratitis, dacrocystitis, endophthalmitis, and panophthalmitis during the 2-year period (July 2012–December 2014) from a total of 3247 specimens received for microbial culture at L & T Microbiology Research Centre, Kamaliyavan Bajaj Research Centre, Sankara Nethralaya, a tertiary eye care center at Chennai, India. Study protocol was approved by the institutional ethics sub-committee (IRB). After the ocular examinations, using standard microbiological techniques, specimens for culture and smear were obtained (7, 8).

(ii) Identification of Pseudomonas aeruginosa isolates

The different ocular specimens submitted to L & T Microbiology Research Centre were cultured on blood agar, MacConkey agar and Brain Heart Infusion Broth (BHIB) and they were incubated at 37 °C for 24 hours and on Mueller Hinton agar plates to assess pigment production. The culture plates were processed using standard microbiological procedures, Characterization and identification of P. aeruginosa was carried out using a combination of colonial morphology, Gram stain characteristics, motility tests, pigmentation, oxidation-fermentation tests, catalase and oxidizer activity tests and pyocyanin production [9]. Detailed distribution of Pseudomonas aeruginosa among the ocular specimens was shown in Table 1.

Phenotypic detection method

We used Standard method of drug susceptibility testing using Muller-Hinton agar and bacterial isolates to be tested (in 0.5 McFarland’s standard concentration), quality control strains Escherichia coli ATCC 35218 (positive control-ESBL), Klebsiella pneumoniae 700603 (MBL-Positive control) and Escherichia coli ATCC 25922 (negative control). One isolate was studied from each patient. The drug susceptibility pattern screened for Pseudomonas aeruginosa strains to Penicillins: ampicillin (10 µg), amoxycillin (20 µg), piperacillin (100 µg); cephalosporins: cephalxin (30 µg), cefuroxime (30 µg), cefazolin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), cefoperazone (75 µg), cefpodoxime 10 µg, cefdinir (5 µg), cefpime (30 µg); Carbapenems: imipenem (10 µg), meropenem (10 µg); Monobactams: aztreonem (30 µg); combinations: ampicillin+ sulphactam (10/10 µg), amoxycillin+clavulanic acid (20/10 µg), piperacillin+tazobactum (100/100 µg); Aminoglycosides: gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg); Quinolones: ciprofloxacin (5 µg), ofloxacin (5 µg), levofloxacin (5 µg), gatifloxacin (5 µg); Tetracyclines: doxycycline (30 µg); Macrolides: azithromycin (15 µg) and miscellaneous: chloramphenicol (30 µg) respectively was investigated by the Kirby-Bauer disk diffusion method according to CLSI guidelines [10].

Mueller-Hinton broth was used as the growth medium. The final inoculum concentration was adjusted to 1.5x10⁸ colony-forming units/ml (cfu/ml) followed by plates were inoculated with swabs submerged in the final inoculum concentration and swabbed over the entire surface of the media. The relevant antibiotic discs were placed over and incubated aerobically at 35-37 °C for 18-24 hours. All antibiotic discs were obtained from Hi-media.

ESBL detection

ESBL test was performed by placing disks of ceftazidime (30 µg), cefotaxime (30 µg), cefotaxime-clavulanic acid (30/10 µg), and ceftazidime-clavulanic acid (30/10 µg) on Muller-Hinton agar (MHA) plates at a distance of 30 mm (center to center) from each other. ESBL production was inferred if the zones produced by the discs with clavulanate (ESBL inhibitor) were ≥25 mm larger than those without inhibitor [11,12].

MBL detection

Phenotypic detection of MBL production (EDTA-IMP) was carried out for imipenem-resistant strains by dissolving 186.1 grams of disodium EDTA·2H₂O in 1000 ml of distilled water, 0.5 M EDTA solution was prepared and adjusted to pH 8 by adding sodium hydroxide. Then, 750 µg/disc was prepared by adding 5 microliters of 0.5M EDTA solution per disc and was dried in the incubator. EDTA-imipenem (IMP) disk plus imipenem disk were placed in a MHA plate containing lawn culture of P. aeruginosa. After 18 h of incubation at 37 °C an organism was considered MBL positive if the inhibition zone diameter increased by ≥7 mm towards the IMP plus EDTA or Meropenem(MER) plus EDTA in comparison to IMP or MER disk alone [11, 12].

RESULTS

The present investigation was carried out to isolate and identify Pseudomonas aeruginosa from ocular specimens. All the Pseudomonas aeruginosa isolates were identified and confirmed based on various characteristics: colony morphology, bluish green pigmentation, non lactose fermenting colonies in macconkey agar, presence of motility, positive reaction for oxidase, catalase, simmon’s citrate medium, nitrate reduction and mannitol sugar test and negative reaction for urease, indole, Methyl Red, Vogues Prosker test, sucrose, lactose and maltose s sugars tests. Of the 3247 samples subjected to culture from August 2012–July 2014 by standard method 276 were positive for bacterial growth, thereby suggesting 8.5% of ocular infections mediated by bacterial pathogens. Out of 276 culture positives, 110 (39.8%) isolates were found to be Pseudomonas aeruginosa. Among, 110 P aeruginosa isolates, 101 (9.18%) specimens were only positive for P aeruginosa infection and 8 (7.27%) isolates were identified with groups of bacterial strains other than P aeruginosa and 1 isolate (0.90%) was found to be presence of fungal growth. The polymicrobial distribution of these 8 isolates were predominantly isolated from 7 (5.45%) corneal scraping, 10 (8.7%) vitreous aspirate, 2 (1.81%) Staphylococcus epidermidis (3), Klebsiella pneumonia (2) and Aspergillus flavus (1) and 2 (1.81%) isolates were identified from eviscerated material with Staphylococcus epidermidis (2).

Of the 110 clinical isolates, the maximum number was isolated from 34 (37.4%) corneal scraping followed by 19 (20.9%) conjunctival swab (significant number of colonies found in any one culture media and BHIB were considered to be positive growth). 11(12.1%) vitreous aspirate and eviscerated material, 10(11%) corneal button, 7 (7.7%) Donor corneal Rim (DCR), 6 (6.6%) contact lens and each 1 (0.91%) from scleral scraping, corneal pus, infective suture, iris tissue, bandage from contact lens, conjunctival scraping and intraocular lens.

Out of 110 P aeruginosa tested, drug susceptibility rate was higher for beta lactam group of drugs such as 109 (99.0%) Aztreonam, 104 (94.5%) Ceftepime, 100 (90.9%) for both Meropenem and cefdinir, 99(90%) Imipenem and drug and the least susceptibility rate for 70 (63.6%) piperacillin followed by 76 (69.0%) amoxicillin and 80 (72.7%) cephalexin. Aminoglycosides susceptibility was 96 (87.2%) amikacin followed by 83 (74.5%) tobramycin and 76 (69.0%) gentamicin. Quinolones/Fluoroquinolone susceptibility was 96 (87.2%) for both ciprofloxacin and gatifloxacin followed by 83 (74.5%) moxifloxacin and 82 (74.5%) for both norfloxacin and ofloxacain (Graph 1).

Results for ESBL & MBL test

In ESBL confirmatory-cephalosporin/clavulanate combination disc test, Totally 15 (13.63%) out of 110 isolates displayed positive results against cefotaxime with clavulanic acid compared to cefotaxime alone and cefotaxime with clavulanic acid compared to cefotaxime alone. Among, 15 isolates 5 (5.5%) belonged to corneal button and 4 (4.4%) from corneal scraping isolates, 3 (3.3%) donor corneal rim and three each from Eviscerated material, vitreous aspirate, conjunctival swab respectively. MBL screening test using carbapenem-EDTA combination test showed 11 (10%) out of 110 positive results against Imipenem with EDTA compared to Imipenem alone and/or meropenem with EDTA as compared to meropenem alone. Out of 11 MBL positive isolates, corneal button 3 (2.72%), corneal scraping 4 (4.4%), donor corneal rim 2 (1.81%) and eviscerated material2 (1.81%). The co-existence of both ESBL and MBL were identified in 7 (6.36%) out of 110 isolates consist of corneal scraping 3 (2.72%), corneal button 2 (1.81%) and each 1 (0.91%) from donor corneal rim and eviscerated material (table 1, Graph 2).
Fig. 1: ESBL confirmatory test-Double disk diffusion method. The inhibition zone around the CAC disk is apparently larger than around the CA disk, indicating ESBL production. But no comparable result is obtained with CEC disk and CE disk. 
Abbreviations: CA, Ceftazidime (30 µg); CAC, Ceftazidime+clavulanic acid (30/10 µg); CE, Cefotaxime (30 µg); CEC, Cefotaxime+Clavulanic acid (30/10 µg)

Fig. 2: Carbapenem-EDTA Combination disk method for MBL detection. The inhibition zone around the IPM+EDTA and MRP+EDTA disk is apparently larger than around the IPM and MRP disk respectively, indicating MBL production. 
Abbreviations: IPM, Imipenem (10 µg); IPM+EDTA, Imipenem (10 µg)+5 µl 0.5M EDTA; MRP, Meropenem (10 µg); MRP+EDTA, Meropenem (10 µg)+5 µl 0.5M EDTA

Graph 1: Antimicrobial susceptibility pattern of Pseudomonas aeruginosa isolated from ocular infections

Graph 2: Specimen wise distribution of P. aeruginosa and its beta-lactamases-ESBL and MBL mechanism detected by phenotypic method
DISCUSSION
Keratitis and other ocular infections mediated by *Pseudomonas aeruginosa* is a significant cause of ocular morbidity and permanent blindness. Henceforth, it should be considered as an emergency and requires careful and urgent attention to initiate appropriate therapy [13]. The present study was initiated, due to increased rate of MDR *P. aeruginosa* encountered in ocular specimens. Currently, ESBL and MBL production were not screened routinely in microbiology labs due to cost and other factors. Despite, genetic analysis was standard for MBL production, due to equipments required for PCR analysis which is not affordable to all basic microbiological labs. Hence, we carried out the carbapenem-EDTA combination disk method for identification of MBLs among *P. aeruginosa* recovered from ocular isolates.

In the current study *P. aeruginosa* was predominantly isolated from corneal ulcer 34 (30.9%). Trauma or infection was found to be the major risk factor for the development of *P. aeruginosa* keratitis followed by pre-operative conjunctival swab 19 (17.27%). Contact lens (6 (5.45%)) wearer does not emerge as an important risk factor, though it is noted as a predominant cause for majority risk factor for the development of keratitis. Henceforth, it should be considered as an emergency and requires careful and urgent attention to initiate appropriate therapy and hence mostly urban population hence their care of Contact lens usage and lens (6 (5.45%)) wearer does not emerge as an important risk factor reported a higher resistant rate among isolates [18-20]. The present study was initiated, due to increased rate of MDR *P. aeruginosa* encountered in ocular specimens. Currently, ESBL and MBL production were not screened routinely in microbiology labs due to cost and other factors. Despite, genetic analysis was standard for MBL production, due to equipments required for PCR analysis which is not affordable to all basic microbiological labs.

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The emergence of antibiotic resistant bacteria increases the patients stay at hospital, thus leading to an increased economic burden on them. In the present study, the antibiogram of the 110 isolates of *P. aeruginosa* showed resistance against piperacillin [36.36%], amoxicillin [29.09%], ampicillin and cephalaxin [27.27%], cefoxime [26.36%] though amoxicillin, ampicillin were not used in ocular treatment, we screened those drugs in order to evaluate susceptibility pattern of *P. aeruginosa* from ocular infection with other infections. Among the drugs commonly used for treating ocular infections, increased resistance was noted against Gentamycin [23.63%], Gatifloxacin [20.9%], Moxifloxacin [20%], Tobramycin [20%], ciprofloxacin [19.09%]. Our findings were similar to the observations by Ibukun et al., who reported a higher susceptibility [15]. Our findings differed from those of Dwivedi et al., Arya M et al. who reported a higher resistant rate among isolates obtained from post operative wound infections [16, 17]. Result of this study, shows that *P. aeruginosa* from ocular isolates were more susceptible to newer fluoroquinolones, amikacin, Ciprofloxacin, Gatifloxacin and Moxifloxacin. However resistance rates is gradually emerging among *P. aeruginosa* against gentamicin and moxifloxacin. These findings may show less similar to other researchers (Hakeem et al., 2011, Ferguson et al., 2007) compared with our results [18-19], which is due to the isolate of our study included only ocular specimens, whereas most of the study includes wide group of specimens such as blood, urine, pus and etc. Studies with ocular isolates by [Malathi et al., 2002 and 2001] results were concordance with our findings [8,20].

Our study showed that among 110 *Pseudomonas aeruginosa* isolates, 15 [13.63%] were ESBL producers, which was nearly similar to 20.27 % and 22.2% ESBL producing isolates of *P. aeruginosa* reported by Aggarwal et al., Peshattiwar PD et al. [12,21]. The present study, imipenem and meropenem showed good antipseudomonal activity. A similar observation was made by Jaykumar S and Peshattiwar PD et al. [12,24], while a higher degree of carbapenem resistance was noted by Varaya et al. [25%] [25]. This difference could be attributable to the study environment and nature of ocular specimens.

The most concerning development in recent years has been the emergence of carbapenemases among MDR *P. aeruginosa*. It is alarming to the scientific community that resistance to imipenem [8,18%] and meropenem 10(9.09%) resistance is observed among ocular *P. aeruginosa*. Out of 110 *P. aeruginosa* isolated, 11 [10%] isolates were found to be metallo β lactamases producers. The prevalence of MBLs in the present study was consistent with findings of Ibukun et al., Navaneeth et al., Peshattiwar PD et al., and others [12,15,26]. In the present study, 5 isolates [45.45 % out of 11] MBLs were resistant to all the antibiotics tested except aztreonam and one strain from eviscerated material showed resistance to all drugs including aztreonam. The presence of MBLs in the pandrug resistant isolates was already observed by Jaykumar S [24]. But emerging scenario of *P. aeruginosa* with pandrug resistant strain(s) among ocular infections is an alert for scientific community which may leads to blindness.

In conclusion, the emergence of ESBL and MBL among *P. aeruginosa* strains is noted in ocular setup. Earlier detection of co-existence of ESBL and MBL production by MDR *P. aeruginosa* may pave the way for appropriate therapy. Further, our study underlines the unique problem of ESBL and MBL, mediated resistance, which has created a therapeutic challenge for the clinicians and microbiologists. To overcome the problem of emergence and the spread of multidrug resistant *P. aeruginosa*, a combined interaction and cooperation...
between the microbiologists, clinicians and the infection control team is needed. So, efforts should be aimed at detecting such resistant bacteria along with their resistance mechanisms, controlling infections caused by them, and finally, providing better alternative therapies against these recalcitrant organisms.

ACKNOWLEDGEMENT

We thank the Indian council of medical research (ICMR), the funding agency, for their generous funding and support for the research work (project code AMR/10/2011-ECD-I).

CONFLICT OF INTERESTS

Declared None

REFERENCE


