Bangladesh J Med Microbiol 2018; 12 (1): 15-19
Bangladesh Society of Medical Microbiologists

Original Article

Phenotypic and Molecular Detection of Extended-spectrum beta-lactamase among Ceftazidime Resistant *Pseudomonas aeruginosa* Isolated from Wound Swab.

Sharmin Sultana[1], Shikha Paul[2], Akhtarun Naher[2], Rahima Akter[2], Tarina Khan[2].

[1]Department of Microbiology and Pathology, Saphena Women's Dental College, Dhaka.
[2]Department of Microbiology, Sir Salimullah Medical College, Dhaka.

Abstract

ESBL producing *Pseudomonas aeruginosa* has been reported to be an important cause of nosocomial infection. The study was undertaken to determine ESBL producing *Pseudomonas aeruginosa* by phenotypic method and to detect bla OXA-10 ESBL gene by molecular method. A total of 288 wound infection cases attending the in-patient department of Mitford Hospital and Dhaka Medical College Hospital were enrolled for this study. *Pseudomonas aeruginosa* was isolated following standard procedure and subjected to antimicrobial susceptibility test by disc-diffusion method. Ceftazidime resistant strains were confirmed by MIC by agar dilution method. Confirmed ceftazidime resistant strains were tested for ESBL production by CLSI phenotypic confirmatory disc diffusion test (PCDDT) and *bla* OXA-10 ESBL gene was identified by employing conventional PCR. Out of 92 *Pseudomonas aeruginosa*, confirmed ceftazidime resistant strains were 60. PCDDT detected 49 (81.67%) ESBL producers and PCR detected 44 (73.33%) positive strains for *bla* OXA-10 ESBL gene among 60 ceftazidime resistant strains of *Pseudomonas aeruginosa*. The study showed high proportion of ESBL producers among ceftazidime resistant *Pseudomonas aeruginosa*.

Key words: *Pseudomonas aeruginosa*, Extended-spectrum beta-lactamase (ESBL), Phenotypic confirmatory disc diffusion test (PCDDT), *bla* OXA-10 gene.

Introduction

Pseudomonas aeruginosa is a Gram negative bacillus of clinical significance and the reason of severe infections in patients with diseases including cystic fibrosis, major surgery, burn, diabetes, cancer and deliberate immuno-suppression.[1] Infections with *Pseudomonas aeruginosa* are life-threatening and hard to treat because of development of resistance against many antimicrobials.[2,3] Development of resistance against antimicrobials in *Pseudomonas aeruginosa* is multifactorial. The innate resistance to many antimicrobial is generally due to its low outer membrane permeability, multidrug efflux pumps, production of inducible AmpC chromosomal β-lactamase and acquired resistance is due to the production of various plasmid-mediated β-lactamase enzymes like extended-spectrum β-lactamases (ESBLs), metallo β-lactamases (MBLs).[2,4] ESBLs are plasmid mediated enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins and monobactams but do not affect cephamycins or carbapenems.[5] Various Ambler's class A ESBLs such as TEM-, SHV-, PER-, VEB- type and class D ESBL such as OXA- type ESBL have been found in *Pseudomonas aeruginosa*.[6,7,8,9] OXA-type ESBLs are predominant in *Pseudomonas aeruginosa* and most OXA-type ESBLs have been derived from OXA-10 (OXA-11, OXA-14, OXA-16 and OXA-17).[7]

The present study is designed to identify *Pseudomonas aeruginosa* from wound swab and to detect the presence of ESBL phenotypically by PCDDT and alongside genotypic detection of *bla* OXA-10 ESBL gene by conventional polymerase chain reaction (PCR).

Materials and Methods

Sample size

A cross sectional study was carried out in the department of Microbiology in Sir Salimullah Medical College during the period of January, 2016 to December, 2016. The research protocol was approved by Ethical Review Committee of Sir Salimullah Medical College and Mitford Hospital. 288 wound infection cases attending the in-patient department of Surgery and Burn unit of Sir Salimullah Medical College and Mitford Hospital and also Burn and Plastic Surgery unit of Dhaka Medical College Hospital irrespective of age, sex and antibiotic

Correspondence:
Sharmin Sultana
Assistant Professor
Department of Microbiology and Pathology
Saphena Women's Dental College, Dhaka.
Cell: +8801823700527, E-mail: dr.sharmin527@gmail.com

use were enrolled for this study. Among 288 samples, 174 were burn wound swab, 78 samples were surgical wound swab and 36 were diabetic wound swab. Clean surgical wounds without sign of infection were excluded for this study.

Sample collection

Wound swab samples were collected by sterile cotton tipped swab moistened with sterile saline. During collection, the swab was taken by zigzag pattern by rotating the swab stick across the wound without touching the surrounding wound edge or skin. The specimens were immediately kept in a sterile test tube, capped properly and labelled. Then swabs were transferred to microbiology lab without any delay.

Isolation and identification of *Pseudomonas aeruginosa*

All wound swabs were inoculated onto Blood agar media and MacConkey agar media and were incubated aerobically at 37°C for 18-24 hours. Pale colonies on MacConkey agar media, Gram-negative bacilli on Gram staining, pink-red slope and butt without production of gas and H_2S on KIA media, motile, negative urease and indole on MIU media, positive citrate and oxidase test were identified as Pseudomonas spp.[10] Suspected *Pseudomonas* spp. were subcultured on Cetrimide agar media and incubated at 37°C for 18-24 hours for confirmation of *Pseudomonas aeruginosa*. Cetrimide agar media is a selective media for *Pseudomonas aeruginosa*.[11]

Antimicrobial susceptibility test

Antimicrobial susceptibility test was done by Kirby-Bauer modified disc diffusion technique according to the CLSI guidelines[12]. The antibiotics tested were Gentamicin (10rg/disc), Amikacin (30μg/disc), Imipenem (10μg/disc), Meropenem (10μg/disc), Ceftazidime (30μg/disc), Cefepime (30μg/disc), Ciprofloxacin (5μg/disc), Levofloxacin (5μg/disc), Piperacillin-tazobactam (100/10μg/disc), Aztreonam (30μg/disc), Colistin (10μg/disc) and Polymyxin B (300 units/disc) from Oxoid Ltd, UK. *Pseudomonas aeruginosa* ATCC 27853 was used as negative control.

Minimum inhibitory concentration (MIC) of ceftazidime resistant strains

65 ceftazidime resistant strains detected by disc diffusion technique were subjected to MIC by agar dilution method[13] for confirmation of resistance. To prepare ceftazidime stock solution, 250 mg ceftazidime base powder was dissolved in 5ml distilled water. Then 50 ml sterile Mueller-Hinton agar (MHA) was impregnated with 8μl, 16μl and 32μl of ceftazidime stock solution with the help of micropipette tips to achieve working concentration of 8μg/ml, 16μg/ml and 32μg/ml respectively. To obtain 10^4 CFU/spot on the agar surface, 1μl of 10 times diluted 0.5 McFarland turbidity of test inoculums were placed on ceftazidime impregnated MHA plate. The plate was observed after overnight incubation at 37°C. The lowest concentration of ceftazidime impregnated MHA showing no visible growth on agar medium was considered as MIC of ceftazidime of that strain of *Pseudomonas aeruginosa*. Confirmed ceftazidime resistant strains detected by MIC were subjected for phenotypic detection of ESBL and molecular detection of *bla* OXA-10 ESBL gene by conventional PCR.

Detection of ESBL by phenotypic method

ESBL production was evaluated phenotypically by CLSI phenotypic confirmatory disc diffusion test (PCDDT).[12] 0.5 McFarland standard of each isolates was spread on MHA plate. Then Ceftazidime (30μg/disc) and ceftazidime/clavulanic acid (30/10μg/disc), cefotaxime (30μg/disc) and cefotaxime / clavulanic acid (30/10μg/disc) from Himedia Ltd, Mumbai, India were placed at a distance of 30 mm apart from center to center and then incubated overnight at 37°C. A ≥ 5 mm increase in zone diameter for either antimicrobial agent combined with clavulanic acid versus the zone diameter of the agent alone inferred the presence of ESBL production.

Detection of *bla* OXA-10 ESBL gene by PCR

DNA extraction

Few isolated bacterial colonies were taken from MHA media, inoculated into test tube containing Tryptone soy broth and incubated at 37°C for 24 hours. Then it was centrifuged. Supernatant was removed and pellets were transferred into an eppendorf tube for DNA extraction. DNA was extracted from bacterial pellets by boiling method.[14] Extracted DNA was used for amplification of DNA by PCR. PCR was done by using specific primers for detection of *bla* OXA-10 ESBL.

DNA amplification

Primer sequence for *bla* OXA-10 ESBL gene[15]

Forward primer, OXA-10 F: 5'- ATTATCGG-CCTAGAAACTGG -3' Reverse primer,OXA-10 R: 5'-CTTACTTCGCCAACTTCTCTG -3' Product size: 170 bp Ladder: 100bp

Visualization and interpretation of results

The gel was observed under UV trans-illuminator for DNA bands. The DNA bands were identified according to their molecular size by comparing with 100bp molecular weight marker loaded in a separate lane. Sample showing the presence of corresponding 170 bp bands were considered positive for presence of *bla* OXA-10 ESBL gene.

Results

Culture of 288 wound swab yielded 229 (79.51%) organisms. Among 229 isolates, 149 (85.63%) were from burn wound swab, 46 (58.97%) were from surgical wound swab and 34 (94.44%) were from diabetic wound swab (Table-I).

Table-I: Distribution of bacteria isolated from different types of wound swab (n=288)

Type of samples	Number of samples	Number of isolates		Total
		Gram positive	Gram negative	
Burn wound swab	174	07	142	149 (85.63%)
Surgical wound swab	78	03	43	46 (58.97%)
Diabetic wound swab	36	03	31	34 (94.44%)
Total	**288**	**13**	**216**	**229 (79.51%)**

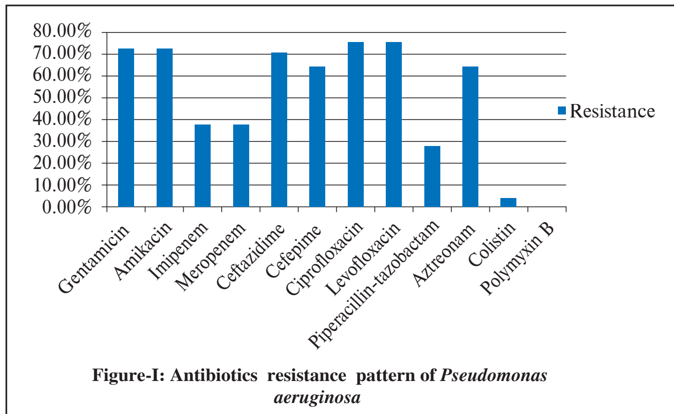

Figure-I: Antibiotics resistance pattern of *Pseudomonas aeruginosa*

Out of 229 isolates, confirmed *Pseudomonas aeruginosa* were 92 (40.17%). In the present study the isolates of *Pseudomonas aeruginosa* showed a pattern of resistance Gentamicin 72.83%, Amikacin 71.74%, Imipenem and Meropenem 38.04%, Ceftazidime 70.65%, Cefepime 65.22%, Ciprofloxacin and Levofloxacin 75.00%, Piperacillin-tazobactam 28.26%, Aztreonam 64.13%, Colistin 4.35% and none of the isolates were resistant to Polymyxin B (Figure 1). Out of 92 *Pseudomonas aeruginosa*, ceftazidime resistant strains were 65 (70.65%) detected by disc diffusion method. The MIC results showed that out of 65, 60 (92.31%) had MIC ≥ 32 µg/ml and 05 (7.69%) had MIC 16 µg/ml. These 60 ceftazidime resistant strains were subjected for phenotypic detection of ESBL and molecular detection of *bla* OXA-10 ESBL gene by conventional PCR. Out of 60 ceftazidime resistant strains of *Pseudomonas aeruginosa*, PCDDT identified 49 (81.67%) ESBL producers and PCR detected 44 (73.33%) *bla* OXA-10 ESBL gene producers among 60 ceftazidime resistant strains of *Pseudomonas aeruginosa* (Table-II).

Table-II : Result of phenotypic method and molecular method among ceftazidime resistant *Pseudomonas aeruginosa* for detection of ESBL (n=60)

Methods for ESBL detection	Ceftazidime resistant strains (60)	
	Positive No. (%)	Negative No. (%)
Phenotypic method (PCDDT	49 (81.67%)	11 (18.33%)
Surgical wound swab		
Molecular method for bla OXA-10 gene	44 (73.33%)	16 (26.67%)

49 (100%) and 45 (91.84%) ESBL producing strains of *Pseudomonas aeruginosa* showed sensitivity to polymyxin B and to colistin respectively.

Discussion

In the present study, 288 samples were collected from different types of infected wound. Out of 288 samples, total 229 (79.51%) bacteria were isolated. Out of 229 isolates, confirmed *Pseudomonas aeruginosa* were 92 (40.17%).

Among the isolated *Pseudomonas aeruginosa*, 72.83% were resistant to Gentamicin, 71.74% to Amikacin, 38.04% of each to imipenem and meropenem, 70.65% to Ceftazidime, 65.22% to Cefepime, 75% of each to Ciprofloxacin and Levofloxacin, 28.26% to Piperacillin -tazobactam, 64.13% to Aztreonam, 4.35% to Colistin and 0.00% to Polymyxin B. Abedin[16] reported more or less similar findings like the present study. In the current study, Ciprofloxacin, Levofloxacin, Gentamicin, Amikacin, Ceftazidime, Cefepime and Aztreonam showed more resistance against the isolates of *Pseudomonas aeruginosa* (Figure 1). On the other hand Polymyxin B and Colistin showed more sensitivity against *Pseudomonas aeruginosa.* This study reported decreased susceptibility of the *Pseudomonas aeruginosa* to several antimicrobials probably due to higher usage of these antibiotics.

The present study showed 81.67% ceftazidime resistant strains were ESBL producers detected by PCDDT (Table-II). Velvizhi et al[17] reported 66.66% ESBL producing *Pseudomonas aeruginosa* in India detected by PCDDT. The ESBL producing *Pseudomonas aeruginosa* are increasing with time in Bangladesh which might be due to overuse and misuse of antibiotics.

PCR is the gold standard method for detection of ESBL producers. In this study, 73.33% ceftazidime resistant strains of *Pseudomonas aeruginosa* were positive for *bla* OXA-10 ESBL gene (Table-II) which was more or less similar with the findings of Farzana et al[18] who reported 80% *bla* OXA producing *Pseudomonas aeruginosa* in

Bangladesh. Al-Rubaye *et al*[15] and Ahmed *et al*[19] found 19.4% and 7.1% *bla* OXA-10 producing *Pseudomonas aeruginosa* in Iraq and in Saudi Arabia respectively. This variation might be due to the fact that different regions have different prevalence of resistance determinant genes. In the present study, negative strains of *Pseudomonas aeruginosa* for *bla* OXA-10 ESBL gene might be due to other ESBL responsible genes that were not studied in this study.

Conclusion

The present study showed 81.67% ceftazidime resistant strains of *Pseudomonas aeruginosa* were ESBL producers detected by phenotypic method and 73.33% ceftazidime resistant strains were positive for *bla* OXA-10 ESBL gene detected by molecular method. In this study, Polymyxin B and Colistin were found to be most effective drug for the treatment of infection caused by ESBL producing *Pseudomonas aeruginosa*. The study reflected that the proportion of ESBL producing *Pseudomonas aeruginosa* are increasing in Bangladsesh. Prompt and accurate detection of these strains is so important to prevent their spread and also to guide appropriate use of antibiotics.

Acknowledgments

We thank the faculties and staffs of the Department of Microbiology, Sir Salimullah Medical College, Dhaka for providing Laboratory support to perform this study.

References

1. Osman KM, Alabady MS, Ata NSSM, Ezzeldin NA and Aly MAK. Genotypic characterization of *Pseudomonas aeruginosa* isolated from human and animal sources in Egypt. Zoonoses and Public Health 2010; 57(5): 329-338.
2. Poole K. *Pseudomonas aeruginosa*: resistance to the max. Front Microbiology. 2011; 2: 1-13.
3. Kerr KG and Snelling AM. Pseudomonas aeruginosa: a formidable and ever-present adversary. J Hosp Infect 2009; 73(4): 338-44.
4. Strateva T and Yordanov D. *Pseudomonas aeruginosa* a phenomenon of bacterial resistance. J Med Microbiol 2009; 58(9): 1133-48.
5. Centers for Disease Control and Prevention (CDC). Laboratory detection of extended-spectrum β-lactamases (ESBLs) 2010. Available at URL: https:/ /www.cdc.gov
6. Livermore DM. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin Infect Dis 2002; 34(5): 634-40.
7. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev 2001; 14(4): 933-51.
8. Weldhagen GF. Poirel L and Nordmann P. Ambler class A Extended-Spectrum β-Lactamases in *Pseudomonas aeruginosa*: Novel Developments and Clinical Impact. Antimicrob Agents Chemother 2003; 47(8): 2385-92.
9. De Champs C, Poirel L, Bonnet R, et al. Prospective survey of β-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolated in a French Hospital in 2000. Antimicrob Agents Chemother 2002; 46(9): 3031-4.
10. Cheesbrough M. Microscopical techniques used in Microbiology, culturing bacterial pathogens, biochemical tests to identify bacteria. In Cheesbrough M, editor. District Laboratory Practice in Tropical Countries, Part-2. UK: Cambridge University Press 2006; p. 65, 67-70, 80-84, 132-143, 180, 194.
11. Collee JG and Marr W. Pseudomonas, Stenotrophomonas, Burkholderia. In: Collee JG, Frased AG, Marmion BP, Simons A, eds. Mackie & McCartney Practical Medical Microbiology, 14th ed. USA: Churchill Livingstone 1996; pp.413-424.
12. Clinical and laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Fifth Informational Supplement. CLSI document 2015; M100-S25.
13. Andrews JM. Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy 2001; 48(S1): 5-16.
14. Ercis S, Sancak B and Hascelik G. A comparison of PCR detection of *mec*A with oxacillin disk susceptibility testing in different media and scepter automated system for both *Staphylococcus aereus* and coagulase-negative *Staphylococci* isolates. *I*ndian Journal of Medical Microbiology 2008; 26(1): 21-24.
15. AL-Rubaye DS, Albassam WW, AL-Habobi HM and AL-Rubaye IAHK. Frequency of *blaOxa 10* beta-lactamase gene in *Pseudomonas aeruginosa* isolated from different clinical swab. Iraqi Journal of Science 2015; 56(4C): 3405-3412.
16. Abedin J. In vitro and in vivo evaluation of effect of antibiotic combination against imipenem resistant Pseudomonas aeruginosa isolated from burn wound of DMCH [M.Phil thesis]. Dhaka Medical College, Dhaka 2016; p. 117

17. Velvizhi G, Sucilathangam G and Anna T. Occurrence of Esbl and Mbl in clinical isolates of Pseudomonas aeruginosa-An emerging threat to clinical therapeutics. Indian Journal of Applied Research 2013; p.459-461

18. Farzana R, Shamsuzzaman SM, Mamun KZ and Shears P. Antimicrobial susceptibility pattern of extended spectrum β-lactamase producing gram-negative bacteria isolated from wound and urine in a tertiary care hospital, Dhaka City, Bangladesh. Southeast Asian J Trop Med Public Health 2013; 44(1): 96-103.

19. Ahmed OB, Asghar AH and Bahwerth FS. Prevalence of ESBL genes of Pseudomonas aeruginosa strains isolated from Makkah Hospitals, Saudi Arabia. European Journal of Biology and Medical Science Research 2015; 3(6): 1-7.