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Frequency of extended spectrum beta lactamase producer *P.aeruginosa* strains isolated from burned patients of Motahari hospital, Tehran, Iran

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen which is naturally resistant to a large range of antibiotics like lots of Beta–Lactams (penicillins, cephalosporins and carbapenems) and may cause additional resistance after unsuccessful treatment. The understanding of beta-lactamase identification and detection in these bacteria is very valuable. In recent years a number of variety of new beta-lactamases were detected, apparently as a consequence of the clinical use of novel classes of beta-lactam antibiotics. Thus a reliable test to detect extended spectrum beta-lactamases (ESBLs) in clinical isolates of P.aeruginosa is needed. In this study, a total of 100 clinical isolates of P. aeruginosa were studied to assess sensitivity of P.aeruginosa isolated from burned patients of Motahari Hospital in Tehran and determine ESBL production in them. Antibiogram test was done by disk diffusion method. Beta-lactamases producers were screened by Starch-paper strip method. Further confirmation was done for ESBL producers by double disc test (DDT) and double disc synergy test (DDST). Among the total of 100 isolates that were considered beta-lactamase producer by starch paper strip method, 17% were ESBL positive by DDST, a figure that increased to 70% after imipenem was included. In addition, 20% of isolated P.aeruginosa strains were ESBL positive by other confirmatory test. This study is the first that uses a combination of paper strip method with DDT and DDST.

Key words: P.aeruginosa, ESBL (Extended Spectrum Beta lactamase), DDT (double Disc Test) ,DDST (Double Disk Synergy Test), Paper strip method.

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1. Introduction

Pseudomonas aeruginosa is a member of Pseudomonaaceae family. It is a Gram-negative, aerobic, free-living bacterium and is commonly found in soil and water, generally associated with contaminated water and other skin infections like folliculitis, otitis and corneal ulcers (Dubois, 2008). In fact, P.aeruginosa has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies track its occurrence as a nosocomial pathogen and indicated that antibiotic resistance is increasing in clinical isolates. P.aeruginosa remains the predominant bacterial cause of nosocomial infections in Iran (Lari, 2000). Their resistance to the common antibiotics warrants the continued implementation of national monitoring networks (Poirel, 2003).The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that cannot be infected if the tissue defenses are compromised in some manner. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bone and joint infections, bacteremia, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns, cancer and AIDS who are immunosuppressed. The case fatality rate in these patients is near 50%. In addition to factors involved in the virulence of P.aeruginosa, its resistance to antimicrobial contributes to its role as an effective opportunistic pathogen. Resistance to anti-pseudomonal beta-lactams has been well described and resistance to recent generation of cephalosporins, monobactams and carbapenems is becoming a disturbing clinical problem (Gales, 2001; Ben-Marez, 1999; Medeiros, 1997). Functional classification of Beta-Lactamases shows 4 groups, group 1 consists of cephalosporinases that are not inhibited by clavulanic acid in molecular class C, group 2 includes penicillinases, cephalosporinases, or both inhibited by clavulanic acid, corresponding to the molecular classes A and D, group 3 includes metalloenzyme, molecular Class B which are not inhibited by clavulanic acid and also are able to hydrolyse penicillins, cephalosporins, and carbapenems and finally, group 4 includes penicillinases that are not inhibited by clavulanic acid and don't have yet a corresponding molecular class yet (Bush, 1995).

P.aeruginosa strains presents further difficulties, because it not only has an inducible AmpC enzyme but also has a much greater degree of impermeability than Enterobacteriaceae, as well as efflux-mediated resistance (Livermore, 1995; Cavallo, 2007; Rodriguez, 2009; Lomovskaya, 2001) Enzymes hydrolyzing extended-spectrum cephalosporins of Ambler classes A, B, and D have been reported in P.aeruginosa (Champs, 2002). Various ESBL-screening methods were designed to compare the reliabilities of detecting ESBLs in clinical isolates of P.aeruginosa whose beta-lactamases were well characterized (Jiang, 2006; Glupczynski, 2010) such as double disk tests, E-test ESBL strips, combined disc methods and VITEK ESBL cards (Livermore, 2001). Expression of the AmpC beta-lactamase affects all beta-lactams to various extents, which may lead to the misdetection of other acquired beta-lactamases (Champs, 2002). In this study, we attempted to determine their sensitivity or resistance to common antibiotics and furthermore beta-lactamase producers screened by Starch paper strip method .ESBL producers were confirmed by Modified DDST and DDT.

2. Methodology

2-1. Bacterial strains

A total of 100 clinical isolates of P.aeruginosa were obtained from burned patients from Motahari Hospital, Tehran, Iran, from Jan to Apr 2010.

2-2. Organism identification

All isolates were identified at the Motahari Hospital by the routine methodology. Upon receipt at the monitoring laboratory, isolates were subcultured onto cetrimide agar and Triple sugar Iron Agar (TSI) to ensure their viablity and purity. Special identification was confirmed with the oxidase test (Murray, 1995).

2-3. Antimicrobial susceptibility testing and screening for ESBLs

Antibiogram based on Kirby_Bauer disk diffusion method was used to determine overnight cultures sensitivity or resistant against cefixime (CFM) (5 μg), cefepime (CPM)(50 μg), aztreonam (AO) (30 μg), ceftriaxon (CRO) (30 μg) and cloxacillin (CX) (30 μg) (all were purchased from Himedia, India) by using Mueller-Hinton (MHA) agar (Murray, 1995) (Figure 1: shows the schematic arrangement of antibiotics in antibiogram method Figure2: Shows the array of antibiotic disks in antibiogram test).

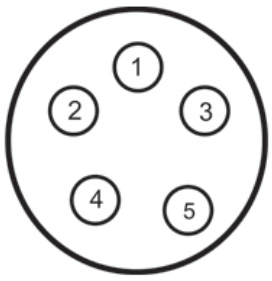

Figure 1. *The model of antibiotics array in Antibiogram test: 1- Cefixime; 2- Cefepime; 3- Aztreonam; 4- Ceftriaxon; 5- Cloxacillin*

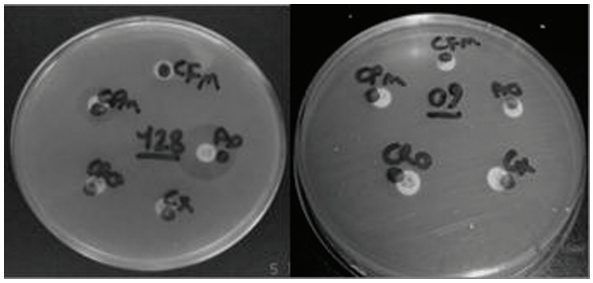

Figure 2. *A clear inhibition zone was detected only against aztreonam, in one of P.aeroginusa isolates.*

2-4. Test for beta-lactamases detection

Based on Paper strip method, to prepare iodometric paper strips, 0.1 g of soluble starch is added to 45 ml of distilled water and dissolved by boiling. 5ml of penicillin G 1000000 Iu/ml was added after cooling. Filter papers (1-5 cm) were soaked in this solution then air-dried for 2 h. The prepared strips were stable for 1 year at -70°C. The prepared strips were moistened in 2% (w/v) aqueous potassium iodine before use and smeared with colonies from an overnight culture plate. Decolorization within 1 min indicated beta-lactamases activity. E.coli ATCC 25922 and K.pneumonia ATCC 700603 were used as negative and positive controls respectively (Cavallo, 1996; Gad, 2007; Oberhofer, 1982; Shobba, 2009).

2-5. Modified DDST and DDT

Based on Lee et al study, the indicator antibiotics for ESBL detection in DDST test are ceftazidim, aztreonam and cefepime (Lee, 2005).In addition, an imipenem disk was used beside the indicator antibiotics to detect group 3 metalo-betalactamases (Bush, 1995).DDST, was performed by placing disks of cloxacillin (CX) (30 μg), cefepime (CPM) (50 μg), ceftriaxone (CL) (10 μg) and ciprofloxacin (CF) (5 μg) at a distance of 20 mm (center to center) from a disk containing AC (amoxicillin, 20 μg, and clavulanic acid, 10 μg) and cefotaxime (CE) (30 μg), Imipenem (I) (10 μg) and aztreonam (AO) (30 μg) at a distance of 20 mm (center to center) from a disk containing cefotaxime+clavulanic acid (CEC) (30 μg+10 μg) (Lari, 2000; Lee, 2005; Poirel, 2004).
ESBL production was inferred if the zones produced by the disks with clavulante were 5 mm larger than those without inhibitor in DDT, another ESBL confirmatory test (Livermore, 1995). In order to conveniently perform these two tests and observe the results, we integrated the DDSTs and combined disk tests into one plate (see Fig. 3 and Fig. 4) but for double time by placing disks of ceftazidime, cefotaxime, aztreonam, cefepime (30 μg each), ceftazidime-clavulanic acid, and cefotaxime-clavulanic acid on MHA plates. As mentioned before the distances of ceftazidime-clavulanic acid and cefotaxime-clavulanic acid, ceftazidime, and cefotaxime, aztreonam, and cefepime from AC were 20 mm. The criteria for result interpretation were the same as those for the DDST and the combined disk test (Glupczyski, 2010; Poirel, 2004; Flavia, 2006; Luzzaro, 2001; Weldhagen, 2003).

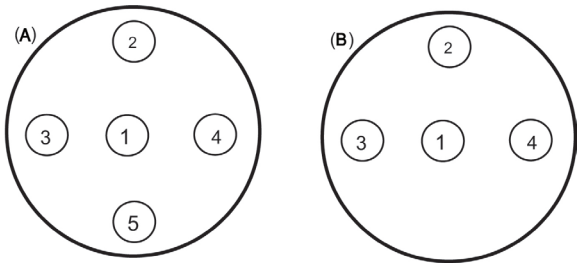

Figure 3. *The model of antibiotics array in DDST and DDT; (A): 1-Amoxiclave; 2- Cloxacillin; 3-Cefepime; 4-Ceftriaxone; 5-Ciprofloxacin; (B) 1-Cefotaxime+clavulanic acid; 2-Cefotaxime; 3-Imipenem; 4-Azteonam.*

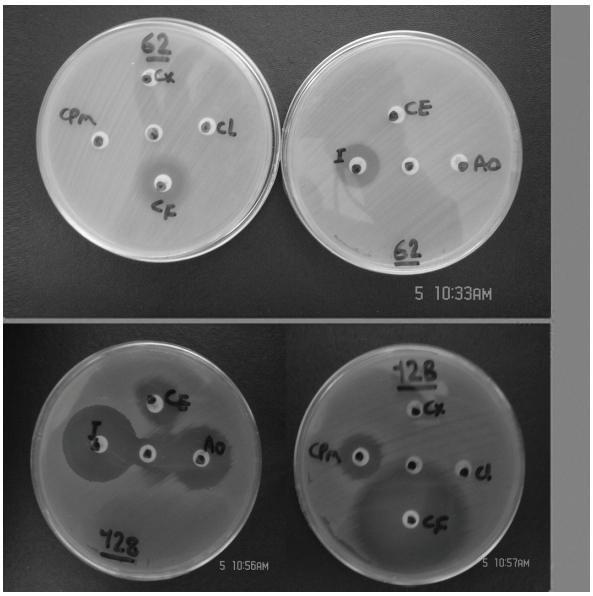

Figure 4. *Double-disk synergy test performed with Amoxiclave, Cloxacillin, Cefepime, Ceftriaxone, Ciprofloxacin, Cefotaxime + clavulanic acid, Cefotaxime, Imipenem and Aztreonam containing disks and producing P. aeruginosa on Mueller-Hinton agar plates. (1) No synergy was visible with containing disk; (2) Synergy was visible with containing disk.*

3. Results

Among the 100 P.aeruginosa strains which were isolated from burned patients of Motahari hospital in Tehran, Iran between January first and April 30th 2010, 100 were resistant to cloxacillin and cefixime, 90% were resistant to ceftriaxon, 83% were resistant to aztreonam and 85% were resistant to cefepime as determined by the disk diffusion method on Mueller-Hinton agar in

the Antibiogram test. Besides, 82% were resistant to cloxacillin, cefexim, ceftriaxon, aztreonamand, cefepime and were classified as multidrug resistant (Table 1 and Fig. 5). The paper strip method showed that beta-lactamases producers in these strains of P. aeruginosa were 100(100%) and all of the strains were positive in this method, however E.coli ATCC 25922 and K.pneumonia ATCC 700603 were used as negative and positive controls, respectively. Of 100 isolated P.aeruginosa strains, 17% were positive in DDST when there was clear inhibition zone for any of the indicator antibiotics by amoxicillin/clavulanic acid, and after addition imipenem it increased to 70% produced class3 beta-lactamases. In DDT, among 100 isolated P.aeruginosa, 20% were positive and showed at least 5 mm difference between cefotaxim and cetotaxim/Glavulanic acid (Fig. 6). Among tested antibiotics, imipenem was the most active drug against P.aeruginosa and ciprofloxacin remained the best floroquinolon against these strains with 34% sensitivity

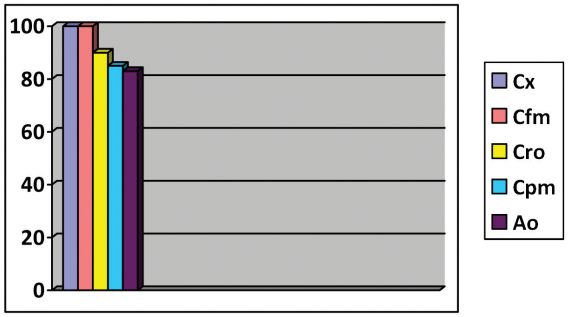

Figure 5. *The result of Antibiogram test: Resistance to antibiotics*

Finally, 7 of 100 isolates strains showed a controversial result, which showed good sensitivity in antibiogram test against aztreonam and cefepim disks but their inhibition zone decreased in DDST.

4. Discussion

ESBL producers have created significant therapeutic problems in the recent years. Thus, there is no doubt that the ESBLs will become increasingly diverse and complex in the future. This will create increasing challenges for those creating guidelines for detection of ESBLs in the clinical microbiology laboratory (Paterson, 2005). On the other hand, ESBL-producing P.aeroginosa has been responsible for numerous nosocomial infections throughout the world and poses challenging infection control issues. Clinical outcomes data indicated that ESBLs are clinically significant and when detected, use of appropriate antibacterial agents will be needed (Cavello, 2001; Rupp, 2003). To show beta-lactamase producer strains, starch-iodine test was done. Beta-lactamase producer rate from this study was similar to the result of Sepehri seresht et al in Iran and both of them showed 100% beta-lactamases producer (Sepehri Seresht, 2007). Further results of this study indicated a high frequency of ESBL Producer P.aeroginosa Strains among Burned Patients of Motahari Hospital (20%) by DDT and (17%) DDST, among them (11%) of isolates were confirmed as ESBL positive by both tests. It must be reminded that DDT and DDST were identified as confirmatory ESBL tests for klebsiella and E.coli spp (Rupp, 2003). 12% of the strains were methallo beta-lactamase producers. Besides, there may be false negative results in DDT and DDST results for P.aeruginosa strains because of AmpC Producers and their much greater permeability compare to enterobacteriacea members (Livermore, 2001). This may be the reason for 7 isolates which showed controversial results in this study.
The occurrence of ESBLs in P.aeruginosa isbeing increasingly reported worldwide recently (Lari, 2000; Gales, 2001; Cavello, 2007). P.aeruginosa isolated in

Table 1. *Antibiotic susceptibility of clinical isolates of p.aeroginosa were obtained from burned patients of Motahari hospital*

	Susceptible		Intermediate		Resistant	
Antibiotic	Number	%	Number	%	Number	%
Cloxacillin(30 µg	0	0	0	0	100	100
Cefixime (5 µg	0	0	0	0	100	100
Ceftriaxone(10 µg	0	0	0	0	100	100
Ciprofloxacin(5 µg	34	34	12	12	54	54
Cefotaxime(30 µg	11	11	8	8	81	81
Cefepime(50 µg	10	10	2	2	88	88
Aztreonam(30 µg	17	17	0	0	83	83
Imipenem(10 µg	70	70	9	9	21	21

the medical practices is often likely to have a nosocomial origin. Although by in vitro tests ESBLs are inhibited by beta-lactamase inhibitors such as clavulanic acid, the activity of beta-lactam/beta-lactamase inhibitor combination agents is influenced by the bacterial inoculums, dose administration regimen and specific type of ESBL present. Like the other study by Gales et al P.aeruginosa isolates showed high resistance rates to most antimicrobial agents tested (Gales, 2002).Recently, carbapenems are regarded as the drug of choice for treatment of infections caused by ESBL-producing organisms. Unfortunately, use of carbapenems has been associated with the emergence of carbapenem-resistant bacterial species such as Stenotrophomonas sp or Pseudomonas sp (Weldhagen, 2003). One earlier study reported that meropenem and imipenem were the most effective antibiotic against P.aeruginosa (Aibinu, 2007; Mirsalehian, 2008) and hopefully in this study imipenem resistant was detected in 21% strains. In this study of 100 isolates, 17(17%) isolates were sensitive to cefepime and aztreonam compared to 29 isolates (34.66%) in Jiang et al study (Jiang, 2006).This difference may be related to the geographic difference between China and Iran and the time of evaluation (2006 V.s to 2009). As it was shown the sensitivity decreased after 3 years in Iran which may be related to increasing drug resistant P.aeroginusa strains. In previous studies in Iran by Hosseinzadegan et al showed 10% ESBL producer strains and as the same in this study11% are ESBL producer(Hosenzadegan, 2005), however the number of ESBL producers in Nigeria was 7.7 % (Aibinu, 2007) and in Latin America is 3.6%(Gales, 2001).
Unfortunately, more recent studies demonstrated the evolution of meropenem-resistant strains of P.aeruginosa (Pournaras, 2005; sahm, 2001). The rate of resistance to aztreonam in France was 42.3% (Cavallo, 1996), in the United States 11.9% (Shawar, 1999), in Spain 23% (Bouza, 1999) and in contrast to Mohajeri's results from Kermanshah/ Iran which showed a high resistance of 80% to aztreonam (Mohajeri, 2001). This is similar to the results of this study (83%). In Flavia's study from Brazil, this rate was 76% (24). Most of our results in this study were very similar to Mohajer's study, except for ciprofloxacin and imipenem resistance rate that showed increases from 38% to 54% and 10% to 21% respectively. This can be related to the time of the study. Indeed, Mohajer's study was done 7 years ago and since then, our results show that Pseudomonas strains have acquired resistant genes (Mohajeri, 2001). ESBLs in P.aeruginosa in Kuwait and other Middle Eastern hospitals may be underestimated because routine detection with a DDST is difficult. Identification of ESBLs is of interest since they confer resistance to all extended-spectrum cephalosporins and aztreonam (poirel, 2001).
The available data from recent study and Mirsalehian's research suggested that imipenem and ciprofloxacin are more effective than cefepime in treating serious infections that involve large numbers of ESBL producing organisms which is in contrast to Aibinu's and Flavia's results (Flavia, 2006; Aibinu, 2007; Mirsalehian, 2008). This difference is related to the different source of Pseudomonas strains from the countries (Iran V.s Nigeria) where theses studies were done.
In conclusion, routine screening for ESBL production need to be done for all pathogens causing complicated infections in burned patients. This study highlights that P.aeruginosa remains an important cause of nosocomial infections and incidence of beta-lactamases producing P.aeruginosa is increasing. Work is undergoing on the molecular study that should increase the precision of the results on beta-lactamase producers.

Acknowledgment

Finally we have to say our thanks for a student grant from Islamic Azad University, Pharmaceutical Sciences Branch during 2009-2010, beside to Motahari hospital lab's personnel for their kindly cooperation and microbiology lab's technicians of Islamic Azad University (IAUPS) for their helps.

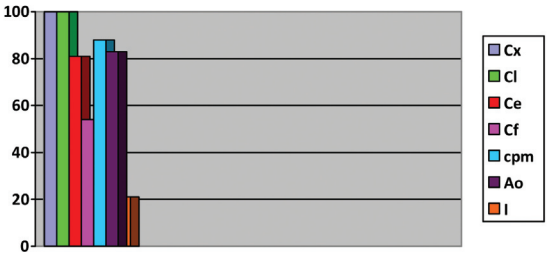

Figure 6. *The result of DDT& DDST: Resistance to antibiotics.*

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