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RECOGNITION OF THE EXTENDED-SPECTRUM-B-LACTAMASES (ESBLS) AMONGST THE GRAM-NEGATIVE BACTERIA ISOLATED FROM BURN WOUND INFECTION

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ABSTRACT

Burn wound infections have caused high rate of death. Gram negative bacteria such as *Pseodomonas auranginosae* and *Acinetobacteria* have been reported as one of the most important bacteria involving in the infections. Besides, different level of resistance to beta-lactam antibiotics have been observed amongst the bacterial strains. Accordingly, the aim of this study was to phenotypically detect the resistance isolates and determine the genes encoding the ESBLs including *tem*, *shv*, and *ctx-m* amongst the resistance isolates at Motahari hospital (Tehran, Iran) by polymerase chain reaction (PCR). Eighty-two isolates were isolated from patients at different wards during 6 months. They were identified as *P. auranginosae* and *Acinetobacter* using biochemical tests. The phenotypic confirmatory test was used to screen the isolates for production of ESBLs. Susceptibility of isolates to 6 different antimicrobial agents was determined using agar disk diffusion method. To amplify the *tem*, *shv*, and *ctx-m* the template DNA was extracted by boiling method. Plasmid DNA was extracted using mini preparation kit and used as template in PCR for detection of *tem*, *shv*, and *ctx-m*. The phenotypic confirmatory assay detected 55 isolates showing resistance against the antibiotics. The rates of resistance to different antibiotics were in the following order: gentamycin (82.75%), amikacin (84.5%), tetracycline (98.2%), ciprofloxacin (88%), ceftriaxone (90%), and ceftizoxime (96.5%). Fifty-five strains harbored the resistance genes so that 31 and 10 isolates had *ctx* and *tem* genes respectively while presence of *shv* gene was not detected amongst the isolates. It appears that TEM, CTX enzymes are the dominant ESBLs among the resistant strains of *P. auranginosae* and *Acinetobacteria* in Iran.

KEYWORDS: ***ESBLs, P. aurangiosa, Acinetobacter, Antibiotic resistance,***

INTRODUCTION

Burn wound infection is one of the main problems to burn patients causing a high percentage of mortality [1]. To treat such infection β-lactam antibiotics are extensively used which leads to pathogen resistance by the production of beta-lactamases (ESBLs). The ESBLs are enzymes that can be produced by bacteria, making them resistant to cephalosporins, for example, cefuroxime, cefotaxime, and ceftazidime, which are the most widely used antibiotics in many hospitals. Subsequently, an indication of the ESBLs production along with the capability of the bacterial strains to deliver the respective genes into other strains have necessitated the recognition of bacteria and the spectrum of the resistance against these antibiotics [2]. Production of beta-lactamases have been the most common mechanism of bacterial resistance [3]. Over 400 different types of ESBLs have been described from clinical samples so far. Many ESBLs are generated by mutations in the genes. Strong selection pressure exerted by antimicrobial use, especially with newer-generation beta-lactam antibiotics, efficiently promotes the ESBL emergence and subsequent spread. It also stimulates further evolution of ESBLs by the accumulation of other mutations with an

astonishing variety of effects on beta-lactamase structure and activity [4, 5]. It has been indicated that gram-negative *Pseudomonas aerunginose* and *Acinetobacter sp.* are the most common pathogens involving in burn wound infection [1]. *P. aerunginosa* is an opportunist, mobile, rod-shaped, and aerobic bacterium which represented high resistance against a range of antibiotics and can use various infections as nutrient sources. *Acinetobacter* sp. is cocci-shaped, obligate aerobic; *A. baumanni* and *A. Lwoffiic* have been reported as the most common species isolating from clinical samples. Since infections by ESBLs production strains turns to be a real problem for the treatment of burn patient, it is necessary to recognize the resistance against beta-lactam antibiotics phenotypically and detect the resistance gene encoding the enzymes such as *tem, shv, ctx* and produce new drugs to tackle the problem. This was a cross-sectional study undertaken to determine the prevalence of ESBL-producing isolates of *P. auranginosae* and *Acinetobacter* at Motahari Hospital and to detect and characterize the *tem*, *shv*, and *ctx-m* genes by polymerase chain reaction (PCR) via the specific primers.

MATERIALS AND METHODS

Sampling

The statistical population of the study consisted of the burned patients of Motahari hospital, Tehran, Iran. 82 samples were chosen during 6 months according to confidence level 95% and coefficient of precision

10% by $\frac{z^2\left(1-\frac{\alpha}{2}\right)p\,(1-p)}{d^2}$ formula.

Bacterial isolation and identification

The microbiological wound swabs were collected from 82 patients with clinical signs and symptoms of burn wound infection. These swab specimens were first inoculated onto Lura and Bertani (LB) medium. The bacterial cultures were then streaked out on LB medium amended with 15% agar to obtain pure culture [6]. *P. aeruginosa* and *Acinetobacter* sp. was biochemically identified by selective media and standard bacteriological methods including colony morphology, gram staining, pyocyanin pigment production, growth at 44°C, catalase and oxidative-fermentative (OF) tests. The isolates were stored in Luria-Bertani broth medium (Merck KGaA, Darmstadt, Germany) containing 30% glycerol at -80°C.

Susceptibility and minimum inhibition concentration (MIC) tests

The antibiotic susceptibility test was carried out for all the isolates on Mueller-Hinton plates and zones of inhibition were measured by Kirby-Bauer agar disk diffusion method in accordance with the recommendations of clinical and laboratory standards institute (CLSI). The antimicrobial agents used in this test were as follows tetracycline (30 µg), ceftriaxone (30 µg), ceftizoxime (30 µg), amikacin (30 µg), gentamycin (10 µg), ciprofloxacin (5 µg), (Mast Diagnostics, Mast Group Ltd, Merseyside, UK). Susceptible *Escherichia coli* was served as control. The results were interpreted as susceptible, intermediately susceptible or resistant by measuring the diameter of inhibition zone, according to the criteria designated by CLSI [7].

Molecular identification of the resistance genes (tem, shv, and stx)

Genomic DNA of the isolates was extracted from 2 ml of cultures acquired from a single colony. The cells were harvested by centrifugation (3000 g, 8 min). Afterwards, the bacterial pellet was suspended in 567 µl of TE buffer (50 mM Tris, 50 mM EDTA, pH 8.0) plus 30 µl 10% SDS and 3 µ1 of proteinase K (20 mg/ml), and incubated for one hour at 37°C. Then 80 µ1 of 10% CTAB in 0.7% NaCl was added, and the mixture was incubated for 10 min at 65°C. The solution was extracted with 750 µl of chloroform/ isoamyl alcohol (24:1), spun, and the aqueous phase was re-extracted with phenol/ chloroform/isoamyl alcohol (25:24:1). DNA was precipitated with 500 µ1 isopropanol from the aqueous phase. The DNA pellet was then washed with 70% ethanol, dried briefly and resuspended in 100 µl of TE buffer. The DNA concentration was determined by measuring the absorbance of the samples at 260 nm using a UV spectrophotometer [8]. The respective resistance genes were amplified by polymerase chain reaction (PCR) using the primers set according to table 1 via a PCR thermal cycler (Biorad, Germany) [9]. Briefly, amplification was performed for 35 cycles, initial

denaturation at 94°C for 5min, denaturation at 94°C for 1min, primer annealing at 55°C, 63°C, and 43°C for 30 for CTX-M, SHV, and TEM respectively, extension at 72°C for 30sec and a final extension at 72°C for 5min. The PCR products were submitted to electrophoresis on agarose gel (1.5%), the molecular size marker (100-bp DNA ladder, BioLabs) was used to determined the DNA expected fragments bands (table 1). The cycling conditions for amplification were as follows: for *shv*, initial denaturation at 95°C for 2 min and 30 cycles of 1 min at 95°C, 30 s at 63°C, and 30 s at 72°C, followed by 5 min at 72°C; for *tem* gene, initial denaturation of 2 min at 95°C and 30 cycles of 30 s at 95°C, 30 s at 43°C, and 1 min at 72°C, followed by 5 min at 72°C; for *ctx-m*, initial denaturation at 95°C for 2 min and 30 cycles of 1 min at 95°C, 45 s at 55°C, and 45 s at 72°C, followed by 5 min at 72°C. The resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels.

Table 1
Characteristics of the applied primers

Primer	Primer sequence	Primer position	Products length	Reference
TEM-52-F	5′ ATAAAATTCTTGAAGAC 3′	1-17	1076	[10]
TEM-52R	5′ TTACCAATGCTTAATCA 3′	1075-1059		
CTX-M-F	5′ TTTGCGATGTGCAGTACCAGTAA 3′	05-227	544	[11]
CTX-M-R	5′ CGATATCGTTGGTGGTGCCATA 3′	748-727		
SHV-F	5′ GCCCGGCTTATTCTTATTTGTCGT 3′	130-151	1016	[12]
SHV-R	5′TCTTTCCGATGCCGCCGCCAGTCA3′	1143-1120		

Statistical analysis

Analysis of the findings was performed using Chi-Square test and Statistical Package for Social Sciences (SPSS, version 16). The study was confirmed by the ethical committee of the Motahari hospital of Medical Sciences.

RESULTS

Sampling

Fifty-eight and twenty-four isolates from hospitalized patients were identified as *P. auranginosa*, *Acinetobacter* respectively as described previously. These isolates were cultured from the clinical specimens including wound (80%), blood (11%), and urine (9%).

Susceptibility test

The isolates represented the most susceptibility against gentamycin (16%), followed by amikacin (12%), tetracyclin (11%), cypropheloxyn (10%), and cefotaxime 1%. Therefore, gentamycin and cefotaxime were the most and least effective antibiotics against the isolates of this study. Table 2 and 3 separately represented the susceptibility against the antibiotics according to *P. auranginosae* and *Acinetobacteria*.

Table 2
Antibiotic susceptibility amongst P. auranginosa

Antibiotic	R		S		I	
	N	Percentage	N	Percentage	N	Percentage
Gentamycine	48	82.75	9	15.5	1	1.75
Amikacine	49	84.5	9	15.5	-	-
Tetracycline	57	98.2	1	1.8	0	0
Ciprofloxacin	51	88	7	12	-	-
Ceftriaxone	52	90	1	1.75	5	8.25
Ceftizoxime	56	96.5	1	1.75	1	1.75

N: number S: sensitive A: intermediate R: resistance

Table 3
Antibiotic susceptibility amongst P. auranginosa

Antibiotic	R		S		I	
	N	Percentage	N	Percentage	N	Percentage
Gentamycine	20	83	4	17	0	0
Amikacine	23	96	1	4	-	-
Tetracycline	8	33.3	8	33.3	8	33.3
Ciprofloxacin	23	96	1	4	-	-
Ceftriaxone	24	100	0	0	0	0
Ceftizoxime	23	96	0	0	1	4

N: number S: sensitive A: intermediate R: resistance

Hereby, the phenotypic confirmatory test (PCT) detected 55 isolates that indicated the different level of resistance against antibiotics including 85.5% (47 out of 55) *Pseudomonas* and 14.5% (8 out of 55) *Acinetobacter.*

Molecular investigation

The isolates exhibiting a resistance against the antibiotics were applied to investigate the presence of the resistance genes (*ctx, tem, shv*). The expected fragments of 1076 bp and 544 bp length relating to *tem* and *ctx* respectively were observed in agarose gel by 100 bp ladder, whereas, the 1016 bp length fragment related to *shv* genes was not observed amongst the selected isolates. 34 (61%) and 13 (39%) isolates of *P. auranginosa* had the *ctx* and *tem* genes respectively. The presence of *tem* and *ctx* genes were confirmed in 7 and 1 of the *Acinetobacter* isolates respectively. The results implied that 25 (60%) and 9 (21%) strains which isolated from infected wound had *ctx* and *tem* genes respectively. The *tem* and *ctx* genes were observed among 4 and 1 strains which isolated from blood respectively. One strain with *ctx* gene was isolated from urine sample.

Statistical results

The results of the chi-square test indicated a significant resistance against ESBLs antibiotics ($P<0.05$). There was also a significant relationship between phenotypic resistance and the genes incidence of *ctx-m* and *tem* genes ($P<0.05$).

DISCUSSION

ESBLs production is not always the bacterial resistance mechanism. An impairment of outer membrane porin channel leads to a reduction of permeability against antibiotics along with efflux mechanism are the non-enzymatic bacterial resistance mechanisms. Therefore, phenotypic methods are not reliable to recognize enzyme producer bacteria. Accordingly, molecular methods using specific primers should be applied to genotypically recognize the enzyme producer bacteria and the type of enzyme. Generally, 82 clinical isolates were evaluated to recognize the susceptibility against beta-lactam antibiotics amongst which 71% were resistance. In the previous study, the incidence of 44.5%, 59%, and 69.56% of ESBLs was reported from Iran [13, 14]. Ref [15] represented that 21.42% of gram-negative isolated bacteria had ESBLs. The incidence of ESBLs is getting momentum; in the USA 3% of isolated bacteria is resistance. In the European countries, the incidence varied from each country; it is below 1% in Netherland, while 40% of *klebsiella pneumoniae* are resistance to ceftazidime in France [16]. A study in Turky indicated that 58% of *klebsiella pneumoniae* were resistance to beta-lactam [17]. In Beiroot a 5 years investigation reported that 20% of *Eshershia coli* were resistance to beta-lactam [18]. Ref [19] isolated 17 Acinetobacter from burned wound which 54% were resistance to tetracycline. However, in our study 79% of *Acinetobacter* isolates were resistance. Ref [20] isolated 149 *Acinetobacteria* from burned patient collecting in Pennsylvania state. The study indicated that 37%, 77%, and 80% of the isolates were resistance to amikacine, gentamycin, and tetracycline respectively. In our study, the percentage was 88%, 83%, and 89% for the respective antibiotics [20]. CTX is one of the ESBLs indicating the most activity against cefotaxime. It varied based on the genetic structure and over 40 types have been identified so far [21]. TEM and SHV are another enzymes with 140 and 60 different types respectively [22]. Recently, CTX beta-lactamase extensively distributed within European countries. For example, the rate of CTX enzymes in France rose from 0.3% to 1.8% between 1999 to 2004. In

England, the rate of resistance to extended-spectrum cephalosporins rose from 1.8% to 7.5% between 2001 to 2004 which most of the resistance mediated by CTX enzymes [23]. The studies showed that most of the resistant strains harboring CTX enzymes which are in accordance with the resistance strains isolating in our study. Hereby, the isolates revealed a significant resistance against beta-lactam antibiotics. The presence of *ctx* and *tem* genes in the resistance strains confirmed the production of ESBLs enzyme as the agent inducing the resistance. Although it has been a while since ESBLs discover for the first time, the clinical importance of the enzyme still remains to be described. Finally, it is recommended to recognize the ESBLs molecularly via PCR techniques to reduce the mortality caused by the pathogens.

REFERENCES

1. Wayne PA. NCCLS, Performance Standards for Antimicrobial Susceptibility Testing: Twelfth Information Supplement M. National Committee for Clinical Laboratory Standards. 2002:100-512.
2. Drieux L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. Clinical Microbiology and Infection. 2008 Jan 1;14(s1):90-103.
3. Khan MA. Bacterial spectrum and susceptibility patterns of pathogens in ICU and IMCU of a secondary care hospital in Kingdom of Saudi Arabia. International Journal of pathology. 2012;10(2):64-70.
4. Jacoby GE, Bush KA. Beta-lactam resistance in the 21st century. Frontiers in antimicrobial resistance: a tribute to Stuart B. Levy. ASM Press, Washington, DC. 2005;570.
5. Hsieh CJ, Shen YH, Hwang KP. Clinical implications, risk factors and mortality following community-onset bacteremia caused by extended-spectrum β-lactamase (ESBL) and non-ESBL producing Escherichia coli. Journal of Microbiology, Immunology and Infection. 2010 Jun 1;43(3):240-8.
6. Hanlon SP, Holt RA, Moore GR, McEwan AG. Isolation and characterization of a strain of Rhodobacter sulfidophilus: a bacterium which grows autotrophically with dimethylsulphide as electron donor. Microbiology. 1994 Aug 1;140(8):1953-8.
7. Hsueh PR, Ko WC, Wu JJ, Lu JJ, Wang FD, Wu HY, Wu TL, Teng LJ. Consensus statement on the adherence to Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Testing Guidelines (CLSI-2010 and CLSI-2010-update) for Enterobacteriaceae in clinical microbiology laboratories in Taiwan. Journal of Microbiology, Immunology and Infection. 2010 Oct 1;43(5):452-5.
8. Sambrook J, Fritsch EF, Maniatis T. Plasmid DNA extraction form bacteria and purification. Molecular Cloning: a laboratory manual, Second edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. 1989:1-23.
9. Jemima SA, Verghese S. Multiplex PCR for bla^ sub CTX-M^ & bla^ sub SHV^ in the extended spectrum beta lactamase (ESBL) producing Gram-negative isolates. Indian Journal of Medical Research. 2008 Sep 1;128(3):313.
10. Szych J, Gierczyński R, Wardak S, Cieślik A. WYSTĘPOWANIE I CHARAKTERYSTYKA SZCZEPÓW OPORNYCH NA. Medycyna Doświadczalna. 2005;57(2):115.
11. Coque TM, Oliver A, Pérez-Díaz JC, Baquero F, Cantón R. Genes encoding TEM-4, SHV-2, and CTX-M-10 extended-spectrum β-lactamases are carried by multiple Klebsiella pneumoniae clones in a single hospital (Madrid, 1989 to 2000). Antimicrobial agents and chemotherapy. 2002 Feb 1;46(2):500-10.
12. Leinberger DM, Grimm V, Rubtsova M, Weile J, Schröppel K, Wichelhaus TA, Knabbe C, Schmid RD, Bachmann TT. Integrated detection of extended-spectrum-beta-lactam resistance by DNA microarray-based genotyping of TEM, SHV, and CTX-M genes. Journal of clinical microbiology. 2010 Feb 1;48(2):460-71.
13. Mirsalehian A, Feizabadi M, Nakhjavani FA, Jabalameli F, Goli H, Kalantari N. Detection of VEB-1, OXA-10 and PER-1 genotypes in extended-spectrum β-lactamase-producing Pseudomonas aeruginosa strains isolated

from burn patients. Burns. 2010 Feb 28;36(1):70-4.
14. Tafti A, Zandi H, Vakli M, Mousavi SM, Zarei M. Frequency of β-lactamase and metallo-β-lactamase in Pseudomonas aeruginosa strains isolated from burn wounds in Yazd burn hospital during 2011-2012. KAUMS Journal (FEYZ). 2014 Apr 15;18(2):167-74.
15. Shobha KL, Lenka PR, Sharma MK, Ramachandra L, Bairy I. Metallo-β-lactamase production among Pseudomonas species and Acinetobacter species in costal Karnataka. Journal of Clinical and Diagnostic Research. 2009;3(5):1747-53.
16. Prodinger WM, Fille M, Bauernfeind A, Stemplinger I, Amann S, Pfausler B, Lass-Florl C, Dierich MP. Molecular epidemiology of Klebsiella pneumoniae producing SHV-5 beta-lactamase: parallel outbreaks due to multiple plasmid transfer. Journal of clinical microbiology. 1996 Mar 1;34(3):564-8.
17. Günseren F, Mamıkoğlu L, Öztürk S, Yücesoy M, Biberoğlu K, Yuluğ N, Doğanay M, Sümerkan B, Kocagöz S, Ünal S, Çetin S. A surveillance study of antimicrobial resistance of gram-negative bacteria isolated from intensive care units in eight hospitals in Turkey. Journal of Antimicrobial Chemotherapy. 1999 Mar 1;43(3):373-8.
18. Daoud Z, Hakime N. Prevalence and susceptibility patterns of extended-spectrum betalactamase-producing Escherichia coli and Klebsiella pneumoniae in a general university hospital in Beirut, Lebanon. Rev Esp Quimioter. 2003 Jun;16(2):233-8.
19. Stevenson KB, Samore M, Barbera J, Moore JW, Hannah E, Houck P, Tenover FC, Gerberding JL. Detection of antimicrobial resistance by small rural hospital microbiology laboratories: comparison of survey responses with current NCCLS laboratory standards. Diagnostic microbiology and infectious disease. 2003 Sep 30;47(1):303-11.
20. Adams-Haduch JM, Paterson DL, Sidjabat HE, Pasculle AW, Potoski BA, Muto CA, Harrison LH, Doi Y. Genetic basis of multidrug resistance in Acinetobacter baumannii clinical isolates at a tertiary medical center in Pennsylvania. Antimicrobial agents and chemotherapy. 2008 Nov 1;52(11):3837-43.
21. Pitout JD, Hossain A, Hanson ND. Phenotypic and Molecular Detection of CTX-M-β-Lactamases Produced by Escherichia coli and Klebsiella spp. Journal of clinical microbiology. 2004 Dec 1;42(12):5715-21.
22. Midolo PD, Matthews D, Fernandez CD, Kerr TG. Detection of extended spectrum β-lactamases in the routine clinical microbiology laboratory. Pathology. 2002 Jan 1;34(4):362-4.
23. Poirel L, Naas T, Le Thomas I, Karim A, Bingen E, Nordmann P. CTX-M-type extended-spectrum β-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrobial agents and chemotherapy. 2001 Dec 1;45(12):3355-61.