Determination of the frequency of *blaCTX-M, blaSHV* and *blaTEM* genes in *Esherichia coli* isolated from Burns patients in Tehran Shahid Motahari Hospital

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Abstract

The genes blaSHV, blaTEM, blaCTX-M of the beta-lactamase enzymes are located on transportable elements. The aim of this study was to determine the abundance of Escherichia coli producing ESBL, lactamase genes of blaSHV, blaTEM, blaCTX-M by Multiplex PCR and their relationships by creating antibiotic resistance in strains of *E. Coli*. Clinical samples were collected from burns patients hospitalized at Motahari hospital of Tehran in the last quarter of 2015. Bacteria were identified by standard biochemical methods. Antibiotic sensitivity of E. Coli against common antibiotics in the pharmaceutical market of Iran,was determind by Kirby – Bauer method. DNA was then isolated from the bacteria and presence of antibiotic resistant genes was determined by PCR and multiplex PCR. Antibiotic resistance and presence of blaTEM, blaSHV, blaCTX-M genes were determined in 100 clinical strains of *Escherichia coli* and standard strain ATCC 25922.

Findings: In total, 167 urine samples were investigated and after biochemical testing 100 *E. Coli* were isolated from the samples. Antibiotic susceptibility test results indicated that the highest and the lowest resistances were related to Ampicillin (97%) and Nitrofurantoin (11%), Imipenem (0%) respectively. It means none of the isolates was found to be resistant to Imipenem. 63 samples of ESBL were positive, analysis of extension of blaSHV, blaTEM and blaCTX-M genes were 44.4%, 61.90% and 71.42% respectively.

The results indicate that the process of antibiotic resistance is on the rise. Development of strains resistant to several antibiotics (MDR) is a significant health threat. Resistance patterns in each region can vary; therefore, accurate identification of resistant strains of each region, providing antibiogram, education of laboratory staff on the correct interpretation of the results of antibiogram and providing detailed reports to physicians can reduce mortality, reduce treatment costs, prevent failures and reduce the spread of resistance.

Key words: ESBL, Multiplex PCR, blaSHV, blaTEM, blaCTX-M

Introduction

Escherichia coli is one of the most common etiologic agents in nosocomial infections. *E. Coli* is a gram-negative bacillus, optional anaerobic or aerobic, and a member of the family of Enterobacteriaceae which causes diseases such as gastroenteritis, sepsis, meningitis, respiratory system infections, wound infections and especially urinary infection (1,2). Unfortunately, treatment of these infections nowadays is facing many problems and is one of the reasons for the acquisition of encoding plasmids of Extended Spectrum Beta Lactamases or ESBL by bacteria (2,3). Different strategies are used by bacteria to be immune against dangerous effects of antibiotics one of which is production of Beta-Lactamases-enzymes by gram-negative bacteria against beta-lactam antibiotics (4). ESBL-producing bacteria are dispersed all over the world and in addition to causing nosocomial infections, are easily spread in the community and cause one of the most important health problems. The frequency of these bacteria is different in various regions. The responsible gene for resistance to Extended Spectrum Beta Lactamases (ESBLs) is most often located in the plasmid and therefore can spread more rapidly among bacteria (5). There may be genes for resistance to other antibiotics in these plasmids at the same time (e.g. amino glycosides), which leads to simultaneous resistance of bacteria to several antibiotics, thus appropriate medications should be targeted toward these bacteria which in turn will lead to spread of ESBL strains(6). Beta- Lactamases are a class of antibiotics that because of their shared central structure, are in this category (7). Beta-lactam antibiotics operate by preventing and deterring. This causes a breakdown of the cell wall biosynthesis and cell function and as a result causes gelatinous deformation of the cell (8). Micro-organisms produce enzymes that destroy the active drug such as produced beta-lactamase by Gram-negative bacteria (8). Beta-Lactamases are members of hydrolytic enzyme families that by hydrolyzing beta-lactam antibiotics make them become derivatives without antibacterial activity (9). In Gram-negative bacteria, production of beta-lactamase enzymes are widely reported in *Enterobacteriaceae, Haemophilus influenzae, Moraxella, Neisseria gonorrhoeae A, Vibrio cholerae* and *Pseudomonas aeruginosa* (10). These enzymes were first recognized in the 1980s and most of them were of TEM, SHV which have been developed as a result of point-mutations in the main enzymes without extended-spectrum activity. CTX-M family of ESBL were first reported in 1989 in Germany, and then spread around the world. Often these enzymes are reported to be in *Escherichia coli, Klebsiella* but are also seen in other *Enterobacteriaceae* (11). Identifying producing strains of ESBL can provide useful epidemiological information about resistance patterns of microorganisms that causenosocomial infections and guide us in making the right choice of antibiotic(12).By 1999, most of ESBL strains which were isolated from patients consisted of blaTEM and blaSHV but more recently blaCTX-M ESBL has often been isolated from patients, and around the world, members of the *Enterobacteriaceae* containing blaCTX-M gene are being separated (13). Transmission and rapid spread of organisms that are capable of producing these enzymes have led to a higher rate of nosocomial infections around the world (14). Therefore, determination of the frequency of blaTEM , blaSHV and blaCTX-M genes in strains of *E. Coli* blaTEM which are isolated from burns patients to help them in the treatment process and improve the quality and effectiveness of treatment in these patients and detection of resistance pattern of *E. Coli* strains isolated from urine of burns patients for effective treatment and shortening duration of patients, hospitalization are considered as aims of the present investigation.

Methods

During a 3 months period, 167 urine samples from burns patients with urinary infection were transferred from Motahari Hospital of Tehran to Saadat Abad laboratory. In order to reach the 100 E. Coli sample, the samples were on a cultured selective environment of eosin methylene blue (EMB) and plates were incubated at 37°C for 24 hours and through performing biochemical tests such as TSI, SIM, MR / VP, lysine Iron agar, Simon citrate, phenylalanine agar and urea agar, nitrate agar were tested. By use of diagnostic kits of GNA and GNB 100 of E. Coli isolates were identified. The related colonies of Positive E. Coli isolates were stored in TSA ambient temperature of 4°C to be used in the next stage of isolation. Determination of sensitivity and antibiotic susceptibility were tested by antibiogram test through spreading diffusion disc (kriby-Baur) based on the guidelines of the National Committee for Clinical Laboratory Standards (CLSI).(15,16) About 10 various antibiotics discs including gentamicin(10 µg), Ciprofloxacin(5 µg), Tetracycline(30 µg), Ampicillin (10 µg), Nalidixic acid (30 µg), Co-trimoxazole (1.25/23.75 µg), Nitrofurantoin (300 µg), Cephalexin (30 µg),Cefotaxime (30 µg), Ceftazidime (30 µg) , Imipenem (10µg) manufactured ROSCO diagnostica A/S, taastrupgaardssvej 30, DK-2630 Taastrup, Denmark, were used.Hybrid drive tests were provided to identify phenotype of ESBL producing bacteria. In this method mixed discs of ceftazidime-clavulanic acid and cefotaxime-clavulanic acid and cefotaxime and ceftazidime of the Rosco company were prepared. After incubation for 24 hours at 37 ° C the increasing diameter of inhibition zone around cefotaxime-clavulanic acid and ceftazidime-clavulanic acid combination disc were determined to be 5 mm more than the diameter of inhibition zone of cefotaxime and ceftazidime around each disc. DNA was extracted by boiling method and for this purpose from each colony, a loop was dissolved in 500 ml of distilled water into each micro tube, then it was put in the Thermo mixer for 15 minutes at 99°C then put on Micro centrifugation around 13000 rpm for 6 minutes. The supernatant containing the DNA was removed and after qualitative and quantitative analyses DNA was used as template for PCR reaction. ESBL-producing gene identification was performed in presence of three genes that produce beta-lactamase CTX-M and TEM and SHV enzymes by PCR and Multiplex PCR experiments. Reaction mixture was 50 µL,constituents of which are shown in Table 1.

Table 1: Reaction parameters

Rank	Substance	Density	volume µL
1	Buffer	1X	5
2	$MgCl_2$	2mM	1
3	dNTP	0.2mM، 10Mm،stock from	1
4	Primer Forward	0.2mM	1
5	Primer Reverse	0.2mM	1
6	Taq DNA polymerase	1.25 U/µL	0.4
7	Pattern of DNA		1
8	Sterilized distilled water		39.6

Reaction was run in a thermo cycler under conditions shown in table (2).

Table 2: Conditions of reaction temperature and time in thermo cycler

Rank	stages	(°C) temperature			Time (min)		
		SHV	CTX-M	TEM	SHV	CTX-M	TEM
1	Initial denaturation	94	94	94	5	4	5
2	Denaturation	94	94	94	1	1	1
3	Annealing	61	60	58	1	0.5	1
4	Extension	72	72	72	1	1	1
5	Final extension	72	72	72	5	5	5
6	Cycle number	35 cycles					

Table 3: Nucleotide sequence of primers.

Gene	Nucleotide sequences of used primers	length
bla_{CTX-M}-F bla_{CTX-M}-R	5′-TTTGCGATGTGCAGTACCAGTAA-3′ 5′-CGATATCGTTGGTGGCATA-3′	(bp) 214
bla_{SHV}-F bla_{SHV}-R	5′-GATGAACGCTTTCCCATGATG-3′ 5′-CGCTGTTATCGCTCATGGTAA-3′	(bp) 590
bla_{TEM}-F bla_{TEM}-R	5′-ATGAGTATTCAACATTTCCG-3′ 5′-GTCACAGTTACCAATGCTTA-3′	(bp) 847

The product of PCR was electrophoresis for the presence of the desired gene in agarose gel (1%). For this purpose 10λ of each sample was mixed with 1λ loading buffer and was cast in gel wells. Power source was set on 120 volts and after 45 minutes, when the color approached almost to the end of the length of gel, the power was turned off, and the Gel, was extracted from the tank and studied and analyzed in the Gel Doc by using UV radiation.

Results

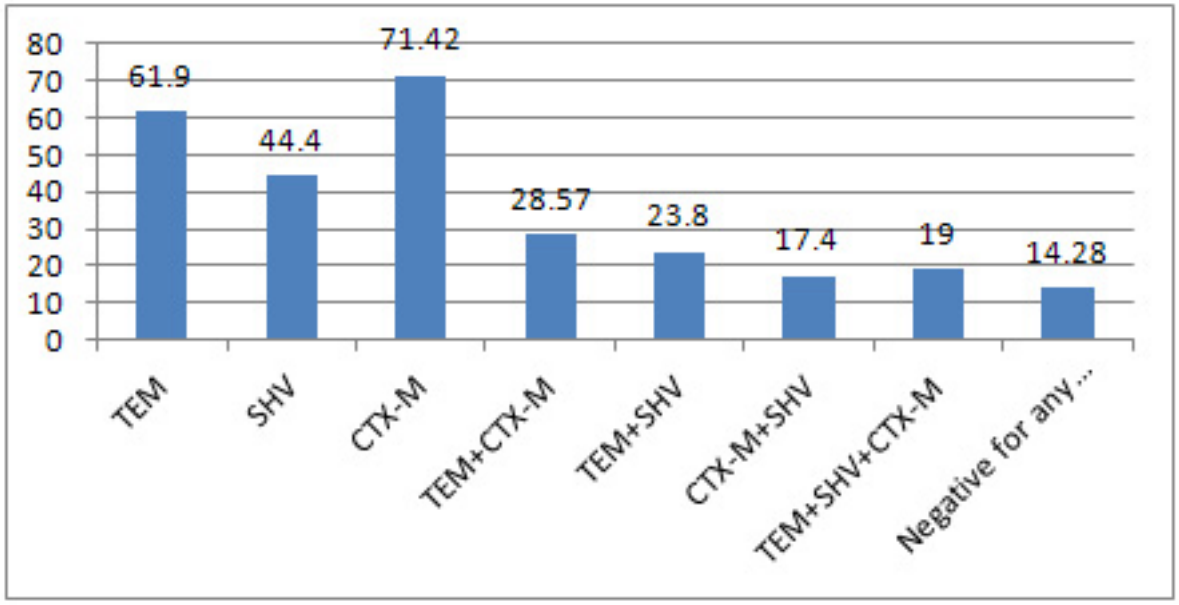
From a total 167 samples and 100 samples of E. Coli isolated from urine of burns patients, with an average age of 16-69 years, 33% of them were men and 67% women. The percentage of sensitivity and resistance of all strains to antibiotics is shown in Table 4 (next page).

Table 4: Sensitivity and resistance to antibiotics

TYPES OF ANTIBIOTICS	NUMBER (%) OF SUSCEPTIBLE STRAINS *SENSITIVE (S)*	NUMBER (%) OF STRAINS WITH INTERMEDIATE SENSITIVITY *INTERMEDIATE (I)*	NUMBER (%) OF RESISTANT STRAINS *RESISTANCE(R)*
Cephalexin	(%5) 5	(%0)0	*(%95)95*
Cotrimoxazole	(%32)32	(%2)2	*(%66)66*
Ampicillin	(%0)0	(%3)3	*(%97)97*
Nitrofurantoin	(%86)86	(%3)3	*(%11)11*
Nalidixic acid	(%42)42	(%12)12	*(%46)46*
Tetracycline	(%33)33	(%0)0	*(%67)67*
Gentamicin	(%16)16	(%52)52	*(%32)32*
Ciprofloxacin	(%62)62	(%20)20	*(%18)18*
Cefotaxime	(% 50)50	(%8)8	*(%42)42*
Ceftazidime	*(%42)42*	(%19)19	*(%39)39*
Imipenem	(%100)100	(%0)0	*(%0)0*

The results of analysis of phenotypic ESBL-producing bacteria showed that 63 samples were positive. After PCR for three beta-lactamase genes, blaTEM, blaSHV and blaCTX-M, the frequency of genes was obtained as in Figure 1:

Figure 1: Frequency of genes



Discussion

Lactamase genes in bacteria, particularly genes of ESBLs, are one of the major factors that increase resistance to beta-lactam antibiotics including extensive-spectrum cephalosporin. Organisms that carry these genes cause increasing morbidity and mortality among patients, thus continuous increase in resistance of these, is a major threat to community health (15 and 16).

In the present study a hundred *E. Coli* strains of 167 urine samples were obtained from Motahhari hospital, Tehran. More than half of all cases of urinary infection were positive for *E.coli.*

In order to evaluate the antibiotic resistance of the strains, antibiogram was performed by Kirby-Bauer method and the percentage of susceptibility and resistance of all strains to antibiotics was evaluated. Results indicate that the greatest reported resistance was against antibiotic ampicillin (97%) and Cephalexin (95%) resistance is noteworthy and reflects use of antibiotics. Such a high percentage of our results is consistent with some of the previous studies but contradicts others. Contradiction could be the result of difference sources of samples, geographical distance, differences in health status of patients and differences in the pattern and antibiotics use table in the different areas (18, 17).

Fujino et al showed that therapeutic regimen with FRPM for patients with acute pyelonephritis caused by ESBL-producing *E. Coli* bacteria is promising (19). In Tamberkar's research resistance to ampicillin (87%) was observed (20) which is consistent with our report. The highest sensitivity to antibiotics nitrofurantoin and ciprofloxacin, have been reported to be 86% and 62% respectively, which is nearly consistent with the results of Moses and his colleagues who reported sensitivity to nitrofurantoin to be 66% (21). In our country the choice of antibiotics for urinary infections are nitrofurantoin and ciprofloxacin.

In this study 3 pairs of oligonucleotide primers for blaSHV, blaTEM and blaCTX-M were used, in which of 100 samples, the presence of E. Coliwas shown as 63 samples of ESBL were positive. Using Multiplex PCR it was shown that CTX-M gene has the highest prevalence (71.42%) consistent with a previous report that is 70% (21) but differentfrom another report that showed 45.2%(23).

The frequency of TEM reported here 61.9%, is close to a previous report 77.6% (24), but different from 8.4% (23) and 32.5% (25,26).

We found SHV gene frequency to be 44.4% which is similar to 34% (21), our results were different from the previous reports (23) and (22), they found no SHV gene. The above differences could be due to origin of samples, presence of other lactamase genes, potency and amount of antibiotics used, country where research was done and different antibiotic resistance mechanisms.

Conclusion

1. Considering the increasing resistance against some antibiotics and differences in effectiveness of antibiotics, implementation of drug susceptibility testing deems appropriate.
2. The high prevalence of antibiotic-resistant E. Coli and high frequency of resistant genes can be proof of indiscriminate use of antibiotics.
3. Use of molecular techniques to accelerate the detection of disease-causing pathogens, especially in urine samples can accurately identify the type of bacteria and thus increase suitabilty of prescribed antibiotic.
4. Appropriate training of hospital staff and proper use of antibiotics by physicians will decrease appearance of drug resistant bacterial strains.

It is recommended that antibiotics only be used when absolutely needed.

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