ABSTRACT

Background and objective: Today, the existence of extended-spectrum beta-lactamases (ESBLs) in the bacteria isolated from children with urinary tract infection is considered a reality and a major health problem in most countries. Since the agent of these infections mostly has a multi-drug resistant, their treatment is sometimes long and difficult. In recent years, beta-lactamases have become one of the biggest factors for failure of antibiotic treatment. Hence, the present research aimed to phenotypically and genotypically study the antibiotic resistance in Klebsiella strains isolated from urine samples of children in Jahrom, Fars Province. Materials and methods: This descriptive-analytic study was carried out in a period of 9 months. Among the 526 isolates from children under school age with urinary tract infection, 216 Klebsiella strains were identified and examined. Their drug susceptibility pattern to 8 antibiotics was studied using antibiotic test by disk diffusion method and then the phenotype confirmatory test was done using combined disks in order to detect ESBL-producing isolates for resistant isolates to cefotaxime and ceftazidime. Phenotypic identification of beta-lactamase genes (CTX-M, SHV, and TEM) was also done using Multiplex PCR. Results: In this study, among the 526 isolates examined, 256 isolates were related to Klebsiella. In antibiogram testing by disk diffusion method, the highest resistance and the highest susceptibility were related to amoxicillin (89.35%) and nitrofurantoin (10.18%), respectively. In the phenotypic confirmatory testing, among the 216 isolates, 109 samples (50.4%) were found to be producers of beta-lactamases. According to the genotypic testing (Multiplex PCR), the frequency of TEM, CTX-M, and SHV genes was 73%, 62%, and 36%, respectively. Conclusion: The findings of the present study suggest the high frequency of Klebsiella strains producing ESBLs in patients under the school age. Rational prescription of beta-lactamases, appropriate infection control policies, and health conditions can reduce the frequency of ESBL-producing bacteria in the urinary tract infections among children.

INTRODUCTION

Klebsiella pneumoniae is one of the most important cases of infection acquired from community or hospital. This bacterium is one of the most common nosocomial pathogens which causes a variety of infections, especially in infants, such as pneumonia, septicemia, diarrhea, abscesses in the liver, endophthalmitis, meningitis, urinary tract infections, and bacteremia [1]. Increased emergence of multi-drug resistance among nosocomial isolates of Klebsiella pneumoniae has limited the treatment options for infections caused by this bacterium. Most of Klebsiella pneumoniae isolates are resistant to multiple drugs (MDR) [2]. Among the mechanisms of antibiotic resistance, beta-lactamases are considered the main defense of Gram-negative bacteria, particularly Klebsiella pneumoniae, against antibiotics of beta-lactam group. According to Ambler, Bush-Jacoby, beta-lactamases are divided into four groups. Bacteria with extended-spectrum beta-lactamase (ESBLs) are resistant to many antibiotics including penicillins, cephalosporin of the first, second, and third generations, and aztreonam (except cephamycins and carbapenems). These enzymes are inhibited by clavulanate. According to the classification of Bush-Jacoby, some of these enzymes are in 2be group (Class A) and others are in 2d group (Class D). The genes encoding these enzymes are placed on mobile genetic elements such as plasmids or chromosomes [1, 2]. Other enzymes found in Klebsiella pneumoniae are Klebsiella pneumonia carbapenemases (KPC) which hydrolyze beta-lactam drugs including monobactams, carbapenems, and third-generation cephalosporins. The gene producing this enzyme is placed on the plasmid and is able to transfer many bacteria. The mortality rate in infections caused by the bacteria containing KPC enzymes is about 50% [1, 3]. The most common ESBLs reported from Western and Asian countries are those derived from TEM, CTX-M, and SHV genes which are located on large plasmids and create drug-resistant strains [4, 5]. Recent studies indicate the expansion and dispersion of beta-lactamase genes in different parts of the world. In studies conducted in Spain and Taiwan, the frequency of ESBLs was reported to be 52.1% and 50%, respectively [6, 7]. Studies carried out in some cities of Iran such as Tehran, Tabriz, Shiraz, Kashan, and Amol showed that the frequency of ESBL-producing strains ranges between 23% and 97.87% [8-12]. The polymerase chain reaction (PCR) is regarded an accurate and highly sensitive method. The present research aims to determine antibiotic resistance pattern and frequency of beta-lactamase genes (TEM, CTX-M, and SHV) in Klebsiella strains producing ESBLs.

Sampling and isolation of bacteria

Using the cross-sectional, descriptive method, 585 cases of urinary tract infection in children (girls and boys) were collected from laboratories of hospitals in Jahrom, Fars Province in a nine-month period from
October 2014 to June 2015 (two seasons). In all cases, demographic and clinical data were recorded in a questionnaire.

The bacteria isolated from patients were inoculated in Skim milk medium containing in 1.5 mL microtubes and then kept at a temperature -20°C. After transferring to the laboratory, bacterial samples were cultured on the selective-discriminative medium of EosineMethylen Blue for purification and then incubated for 24 hours at a temperature of 37°C.

Differential biochemical tests and identification of organisms

The colonies were identified by using the differential biochemical tests including fermentation of sugars in the TSI medium, indole production and motility in the SIM medium, and inhibition in Simon Citrate and Urea Agar media and then comparing the results with standard tables.

Determining the susceptibility and resistance of samples to antibiotics

In order to obtain the antibiotic susceptibility pattern of positive samples, disk diffusion susceptibility test (Kirby-Bauer) on Mueller-Hinton agar medium was used. For this purpose, 8 types of antibiotic disks were purchased from MAST Company (England). These 8 antibiotic disks included gentamicin (10 mg), nitrofurantoin (10 micrograms), nalidixic acid (30 mg), cephalaxin (30 mg), ciprofloxacin (5 micrograms), cefotaxime (15 mg), ceftazidime (2 mg), and amoxicillin (10 mg). The obtained results were recorded based on CLSI Standard Table as susceptible (S), intermediate (I), and Resistant (R).

Isolation of possible strains producing ESBLs

Like the method for determining antibiotic susceptibility by disk diffusion, the medium was prepared and the bacterial suspension was inoculated to the medium. ESBL production was studied by measuring the increase in the diameter of growth inhibition zone around the ceftazidime-clavulanic acid disk or cefotaxime-clavulanic acid disk by 5 mm or more.

Determining the minimum inhibitory concentration (MIC) using E-test

After determining the antibiotic susceptibility of bacteria using disk diffusion method and identification of methicillin-resistant staphylococcus aureus (MRSA). Susceptibility of these strains to vancomycin, teicoplanin, linezolid, and cefidroxil was measured by the E-test method (Liofilchem, Italy) in order to determine the minimum inhibitory concentration (MIC). For this purpose, a suspension of the desired bacterium at a concentration of 0.5 McFarland (1.5 × 10^8) was prepared and cultured on the Mueller-Hinton medium. Then, the E-test strips were put on the inoculated media. The results were controlled after 18-24 hours and interpreted based on CLSI standard. In all phases of the study, standard strains were used as the quality control (QC) of tests. The standard strain of Klebsiella : ATCC: 13883 was used.

Determining the beta-lactamase genes using Multiplex PCR

DNA of isolated Klebsiella strains was extracted using the Cinnagen kit (Iran) based on the protocol specified by the manufacturer. PCR test for amplification beta-lactamase genes (TEM, CTX-M, and SHV) was done using the primers obtained from Cinnagen Company (Table 1).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Amplicon size(bp)</th>
<th>Nucleotide sequence(5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bla-TEM(F)</td>
<td>445</td>
<td>TCGCCGCA1ACAC1ATTC1TCAGAATGA</td>
</tr>
<tr>
<td>bla-TEM(R)</td>
<td>445</td>
<td>AGGCTCACCAGCTCAGATTTAT</td>
</tr>
<tr>
<td>bla-CTX-M(F)</td>
<td>593</td>
<td>ATGTGCAGYACCAGTAARGTKATGGC</td>
</tr>
<tr>
<td>bla-CTX-M( R)</td>
<td>593</td>
<td>TGGGTRAARTARGTSACCAGAAYCAGCGG</td>
</tr>
<tr>
<td>bla-SHV(F)</td>
<td>973</td>
<td>TCTCCCTGTAGGCCACCCTGT</td>
</tr>
<tr>
<td>bla-SHV( R)</td>
<td>973</td>
<td>CCAGTGCAGCAGCTGCAC(C)(C)GT</td>
</tr>
</tbody>
</table>

Thermal cycling for PCR included denaturation for 30 seconds at a temperature of 94 °C, annealing for 30 seconds at a temperature of 60°C, and extension for 2 minutes at a temperature of 72°C. In this study, the standard strain of Klebsiella : ATCC: 13883 was used as the positive control. Finally, PCR products were electrophoresed 1.2% agarose gel medium and then examined under UV light in a transilluminator device [9].

Statistical analysis
After data collection, they were inserted into SPSS-15.5 software and then the frequency tables were plotted. For statistical analysis, Fisher’s exact test, ANOVA, chi-square, and correlation test were used.

Findings

Among the 585 cases of urinary tract infection, 216 isolates of Klebsiella were identified and isolated. Among the 216 samples of Klebsiella, 117 cases (54%) were related to girls and 99 cases (46%) were related to boys. The highest frequency among boys (22.22%) and girls (29.16%) was found in age groups of 2-4 and 4-6, respectively.

The highest drug resistance and susceptibility of isolated Klebsiella strains were against amoxicillin (89.35%) and nitrofurantoin (86.57%), respectively (Table 2).

Table 2: Frequency distribution of Klebsiella isolates by the disk diffusion method based on antibiogram pattern

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Sensitive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>193(89.35%)</td>
<td>8(3.70%)</td>
<td>15(6.94%)</td>
<td>216(100%)</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>22(10.180%)</td>
<td>7(3.24%)</td>
<td>187(86.57%)</td>
<td>216(100%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>133(61.57%)</td>
<td>52(23.11%)</td>
<td>86(36.11%)</td>
<td>216(100%)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>42(19.44%)</td>
<td>10(4.62%)</td>
<td>164(75.92%)</td>
<td>216(100%)</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>181(83.79%)</td>
<td>7(3.24%)</td>
<td>28(12.96%)</td>
<td>216(100%)</td>
</tr>
<tr>
<td>Ceftazidim</td>
<td>137(63.42%)</td>
<td>8(3.67%)</td>
<td>71(32.87%)</td>
<td>216(100%)</td>
</tr>
<tr>
<td>Cefotaxime Sodium</td>
<td>146(67.59%)</td>
<td>10(4.62%)</td>
<td>60(27.77%)</td>
<td>216(100%)</td>
</tr>
<tr>
<td>cephalaxin</td>
<td>84(38.88%)</td>
<td>3(1.38%)</td>
<td>129(59.72%)</td>
<td>216(100%)</td>
</tr>
</tbody>
</table>

Among the 216 samples of Klebsiella, 109 samples were evaluated positive in terms of owning the phenotype of ESBLs. Frequency of ESBL+ in females and males was 29.16% and 21.29%, respectively. Using ANOVA analysis, it was observed that there is a significant relationship between gender and the results of combined disk diffusion based on gender in Klebsiella isolates producing beta-lactamases (P=0.028). The highest and the lowest frequency of isolates producing ESBLs were found in the age groups of 2-4 (17.59%) and under 2 (16.20%), respectively. Using the correlation coefficient test, no significant relationship was found between age group and combined disk test in Klebsiella isolates. The highest drug resistance and the highest drug susceptibility among ESBL+ Klebsiella isolates were related to amoxicillin (44.90%) and nitrofurantoin (37.96%), respectively. MIC test was performed for 109 ESBL+ Klebsiella isolates. Isolates showed a resistance of 100% to both cefazidime and cefotaxime. The frequency of each of the beta-lactamase genes in different antibiotic classes in Klebsiella isolates in all antibiotic classes has been shown (Table 3).

Table 3: Multiple resistance of Klebsiella isolates to different antibiotic classes

<table>
<thead>
<tr>
<th>Nitrofurans (Nitro)</th>
<th>Penicillin (Amox)</th>
<th>Aminoglycosid (Genta)</th>
<th>Fluroquinolones (Cipro)</th>
<th>Quinolones (Nali)</th>
<th>Cephalosporins (Cefa,Cefo,Cefta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>102</td>
<td>35</td>
<td>86</td>
<td>101</td>
<td>109</td>
</tr>
<tr>
<td>0</td>
<td>CTX-M</td>
<td>17</td>
<td>CTX-M</td>
<td>CTX-M</td>
<td>CTX-M</td>
</tr>
<tr>
<td>0</td>
<td>TEM</td>
<td>18</td>
<td>TEM</td>
<td>TEM</td>
<td>TEM</td>
</tr>
<tr>
<td>0</td>
<td>SHV</td>
<td>8</td>
<td>SHV</td>
<td>SHV</td>
<td>SHV</td>
</tr>
<tr>
<td>1</td>
<td>CTX-M/TEM</td>
<td>24</td>
<td>CTX-M/TEM</td>
<td>CTX-M/TEM</td>
<td>CTX-M/TEM</td>
</tr>
<tr>
<td>0</td>
<td>CTX-M/SHV</td>
<td>5</td>
<td>CTX-M/SHV</td>
<td>CTX-M/SHV</td>
<td>CTX-M/SHV</td>
</tr>
<tr>
<td>0</td>
<td>TEM/SHV</td>
<td>7</td>
<td>TEM/SHV</td>
<td>TEM/SHV</td>
<td>TEM/SHV</td>
</tr>
<tr>
<td>25</td>
<td>CTX-M/SHV/TEM</td>
<td>26</td>
<td>CTX-M/SHV/TEM</td>
<td>CTX-M/SHV/TEM</td>
<td>CTX-M/SHV/TEM</td>
</tr>
</tbody>
</table>

CONCLUSION

Antimicrobial resistance has always been considered a serious problem for human health. Infection control is highly beneficial for patients and causes a reduction in mortality rate around the world. The family Enterobacteriaceae, particularly Escherichia coli and Klebsiella, creates a variety of infections in people and especially babies, as 4 million babies die of bacterial infections every year [13]. The highest rate of deaths has been reported to be related to pneumonia, septicemia, meningitis, and diarrhea. It seems that babies are more vulnerable due to incompleteness of their immune system at this age [14]. In this study,
585 cases of urinary tract infection were studied and 216 Klebsiella strains were isolated using the confirmatory tests. Among these 216 strains, 54% (117) were isolated from females, while 46% of them (99) were isolated from males. In studies conducted by Zhanel et al. (2008) in Canada and Kang et al. (2004) in Korea, frequency of isolates was higher in males (59% and 63%, respectively) [15, 16]. In terms of age distribution, patients ranged from 1 month to 6 years. The highest and the lowest isolates were related to the age groups of 2-4 and under 2, respectively. In the study of antibiotic resistance in isolates of Klebsiella, the highest drug resistance and susceptibility were against amoxicillin (89.35%) and nitrofurantoin (86.57%), respectively. In a study conducted by Arkin et al. (2013) on Klebsiella isolates, the highest resistance and the highest susceptibility were found to be related to amoxicillin (100%) and imipenem (1.66%), respectively [10]. Since imipenem and nitrofurantoin are considered nosocomial antibiotics in Iran and are not taken without a prescription, resistance to these drugs is fortunately low in Iran [17]. On the other hand, because of the small molecular size, these two antibiotics can overcome the poor permeability of beta-lactamase in bacteria and especially Pseudomonas species by penetration through purines [18]. Studying the pattern of susceptibility to antibiotics showed that amoxicillin has the lowest antibacterial effect (6.94%). This is also corroborated by other studies [19]. According to the present study, nitrofurantoin and gentamicin are among the drugs which can be used for treatment of patients, if antibiogram is done accurately.

Among the 216 Klebsiella isolates in the urine samples of children, 189 isolates (87.50%) showed multiple drug resistance. Among the 526 samples of Escherichia coli and Klebsiella, 245 cases (47%) were resistant to ceftazidime and cefotaxime or both of them. After performing the combined disk susceptibility test, it was revealed that 136 isolates of Escherichia coli (44%) and 109 isolates of Klebsiella (49%) own the phenotype producing ESBLs. Using the MIC and agar dilution method, the highest production of ESBLs in Escherichia coli in Iran has been calculated to be 96.70% [19]. In a study conducted by Lavign et al. (2007) in France, ESBL enzyme production in Escherichia coli strains has been reported to be less than 3% [20]. The results of a study conducted by Mohammad et al. (2006) in Isfahan showed that ESBLs production in organisms causing urinary tract infection is 80% [21]. In India, Lebanon, and Russia, ESBLs production has been reported to be relatively high (42%, 77.7%, and 15.5%) [22-24]. Comparison of these results shows that the frequency of ESBLs may be different in strains isolated from different countries and event different geographical areas of a country. This depends on sanitary conditions, infection control systems, and treatment methods. The main risk factor for the increase in bacteria producing ESBLs is the overuse of antibiotics (including the third-generation cephalosporins). In studies conducted by Nakhai and Moghadam (2011) in Mashhad, the frequency of positive ESBLs was reported 19% [25]. These figures are less than those obtained in the present study. In studies carried out by Sultan (2011) and Mirsalehian (2010) in Tehran, the frequency of ESBLs isolates was reported to be nearly similar to the results of the present study (56.69% and 59.36%) [26, 27]. In studies conducted in Austria by Eisner et al., ESBL producing isolates showed a growth rate of 58% in the period 1988-2004 [28]. In addition, in a study conducted in 2011 in India and a study by Sasaki in Thailand, the results were close to the findings of the present study [29]. Considering the comparison of different percentages with each other, it should be noted that regional differences in different parts of the world or even within a country create different therapeutic responses to antimicrobial drugs. The origin of these differences in different locations includes genetic differences between individuals, genetic differences between strains, and differences in other fields. Accordingly, treatment methods used in different parts may be different based on the specific features of each region. Therefore, regular and ongoing investigations should to be conducted around the world [30]. For organisms that showed an intermediate resistance pattern to cefotaxime and ceftazidime at antibiogram test and were phenotypically positive in the confirmatory test, MIC (minimum inhibitory concentration) was calculated using E-test. The results showed that 109 ESBL isolates of Klebsiella have a 100% resistance to ceftazidime and cefotaxime. The highest frequency of MIC in Klebsiella isolates for ceftauzime and ceftazidime belonged to 25 micrograms per ml and 12.5 micrograms per ml. In this study, genotype off positive ESBLs was evaluated by Multiplex PCR. According to this method, frequency of TEM, CTX-M, and SHV was 73%, 63%, and 36%, respectively. The most common ESBL found in this study and similar studies [27, 31-32] is TEM. However, many other studies have reported that CTX-M is the most common ESBL [33-38]. There are few studies in which SHV has been introduced as the most common ESBL enzyme [31, 39, 40]. For instance, Paterson et al. reported a wide variety of ESBLs from 7 different countries in 20 years and showed that SHV is the most abundant enzyme among ESBLs (67%) [41]. In this study, frequency of TEM and SHV genes together in positive ESBLs was (8.97%) and less than TEM alone (20%). This is inconsistent with the findings of LAL et al. (2007) in India who reported that the frequency of TEM and SHV genes together and TEM alone was 87.3% and 20%, respectively [42].

In the present study, the highest frequency of TEM in beta-lactamase-producing strains of Klebsiella was related to the age group of 4-6. Among the isolates resistant to cefotaxime and ceftazidime, Multiplex PCR showed that all of them did not contain CTX-M. This is due to the extension of ESBLs scope of action. This means that a bacterium can hydrolyze cefotaxime and ceftazidime by producing ESBL enzymes other than CTX-M. In addition, although the effect of CTX-M is somewhat specific to cefotaxime, it has the ability to hydrolyze other antibiotics of cephalosporin family [43]. In the present study, the extent of resistance of isolates producing ESBLs to nalidixic acid, ciprofloxacin, and gentamicin was relatively high. Some strains were also resistant to nitrofurantoin. However, place of effect and mechanism of nitrofurantoin and gentamicin are different. The results showed that gentamicin and other genes (SHV, TEM, and CTX-M) are significantly related with the resistance of strains producing beta-lactamase to these antibiotics. Genes encoding resistance to these antibiotics are possibly transferred...
with ESBL genes. In a study conducted in the US, among the 20 bacteria resistant to antibiotics isolated from patients in hospitals or nursing homes, 17 bacteria contained 54-kb plasmid which codes resistance to ceftazidime through TEM, ESBL. This plasmid was also the mediator of resistance to gentamicin [44]. In the present study, among the ESBL-producing isolates, those encoding blaCTX-M / TEM / SHV gene showed higher resistance to gentamicin and nitrofurantoin than the isolates encoding other genes. The isolates that simultaneously have all these three genes are more likely to transfer the gene of resistance to gentamicin and nitrofurantoin in plasmid. Confirmation of this require further studies. According to what mentioned above, it is recommended that development of resistance in bacteria to be prevented by monitoring the consumption of antimicrobial agents in the treatment of infections eligible for multiple drug resistance. ESBLs are mostly of plasmid type and since these plasmids are easily transferred between different types of bacteria of Enterobacteriaceae family, accumulation of resistant genes leads to creation of strains with multi-drug resistance. In fact, plasmids, because of their replication that is independent of chromosome, are replicated in large numbers within the cell and cause antibiotic resistance in the case of transfer to other strains [45-48]. Previous studies show the high prevalence of beta-lactamase genes and especially TEM in urinary tract infection. Therefore, in order to identify this type of resistance, it is necessary to apply molecular methods along with phenotypic ones. Patients afflicted with infectious agents producing ESBLs, in addition to lack of treatment by ESBLs, often show resistance to other antibiotics. ESBLs have been of particular importance in treatment strategy, because this is due to treatment failures resulting from prescription of antibiotics without a susceptibility test which itself increases the mortality rate, prolongs the hospitalization period, and increases treatment costs. Therefore, rapid identification of ESBL-producing strains in microbiology laboratories is very important and essential [49, 50]. Production of ESBLs is considered a great threat to the consumption of cephalosporins with extended spectrum. Thus, to treat infections that are suspected to ESBL-producing organisms, the most appropriate antibiotic should be selected very carefully. In addition, strains that their susceptibility to ceftazidime and cefotaxime has reduced should be studied in terms of ESBL genes. Due to the high rate of prevalence of ESBL-producing bacteria in the urinary tract infections, it is recommended to identify the ESBL-producing strains among E. coli and Klebsiella. It is obvious that beta-lactam antibiotics are not recommended for treatment of infection in these patients. In addition, many of these bacteria may be probably resistant non-beta-lactam antibiotics such as nalidixic acid, ciprofloxacin, and gentamicin. In this case, antibiotics such as nitrofurantoin and imipenem are recommended to be applied for treatment.

CONFLICT OF INTEREST
The authors report no conflict of interest

ACKNOWLEDGEMENTS
We appreciate the cooperation of the staff of University of Medical Sciences and Islamic Azad University for their assistance in this study

FINANCIAL DISCLOSURE
None

REFERENCES


[37] Chmelinskis I, Carmeli Y, Leavitt A et al. [2005] CTX-M-2 and a new CTX - M - 39 Enzyme are the major Extended - spectrum Beta Lactamases in multiple Escherichia coli clones Isolated in Tel Aviv, Israel, Antimicrobial agents and chemotherapy. 40(11); 4745 - 4750.


