
Evaluation and Quantification of Resistant Gene in Antibiotic Resistant ESBL Producing *E. coli* in UTI Samples with Diabetes Mellitus: A Molecular Study

Original Research Article

ABSTRACT

In diabetic patients, urinary tract infections (UTIs) are more common and severe often caused by antibiotic resistance and carry worse consequences, due to high blood sugar level and frequent urination. High blood sugar provides favorable environment for the growth of pathogens and some other impairment in immune system, poor metabolic control and incomplete emptying bladder to enhance the risk of UTIs in the diabetic patients. Most of the UTIs are caused by multidrug resistant, Extended Spectrum β lactamase (ESBL) producing bacteria mostly, *Escherichiacoli* (*E. coli*). The prevalence of UTIs caused by ESBL producing Multidrug Resistant (MDR) bacteria is a major global public health concern. The aim of this study was to identify the pattern of multidrug resistance and quantification of resistance genes amongst the *E. coli* isolates which were obtained from UTI patients with diabetes. ESBL producing stains were found 36% by using amoxicillin/clavulanic acid as a β lactamase inhibitor. Out of these stains, following antibiotics resistance rates were achieved with ampicillin, amoxicillin, amikacin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, nitrofurantoin, norfloxacin, tetracycline and amoxicillin/clavulanic acid as 100%, and 0%, respectively. The ESBL encoding genes were characterized for CTX-M, TEM and SHV genes. CTX-M gene was found to have resistance in the *E. coli* isolates, some others genes as TEM and SHV respectively. These characterized genes were analyzed through PCR which is a rapid, sensitive and easy to interpret detection method. Among these isolates, CTX-M was significantly higher 85.4% in the ESBL positive strains ($p < 0.05$). TEM and SHV were found 12.8% and 6.5%, respectively. Early and proper diagnosis is necessary for the management of UTIs in diabetic patients in order to have an adequate treatment.

Keywords: *Diabetes mellitus; urinary tract infections (UTIs); extended spectrum β lactamase (ESBL); multi drug resistant; Escherichia coli.*

1. INTRODUCTION

Diabetes is a globally threat affecting the quality of life and the patients are prone to develop infections compared to the normal population [1], with UTIs being the most common microbial diseases encountered in medical practice affecting people of all ages [2-5], Multiple mechanisms and parameters were found to enhance UTIs in diabetic patients as age, poor metabolic control and high glucose concentration in urine. The high glucose concentration in urine gives favorable growth environment, provides a rich source of nutrients and allows colonization and other foundation for infection. In diabetic patients the maximum morbidity and mortality is caused by infections. According to National Institutes of Health (NIH), an estimated 150 million UTIs are second most infection diagnosed

worldwide every year [6]. American Diabetic Association (ADA) report shows that patients with type 2 diabetes are more likely to have a UTI than patients without diabetes. Out of the 7 million people, 1 million accounts for UTIs in United States in 1999 [7]. Acute UTI is one of the most common acute bacterial infection, over half of adult women have at least one episode in their lifetime [8]. Approximately, 80% of UTIs are caused by uropathogenic *E. coli* in both acquired community and hospitalized patients [9]. Uropathogenic *E. coli* carry a series of virulence markers that enable them to invade, colonize, survive on urinary epithelium and prevent from removal during urination [10-12].

Antibiotic resistance is another serious problem in infections caused by extended spectrum β -lactamases (ESBL) producing uropathogenic *E. coli*. ESBL is a group of enzymes that confers

resistance to most β -lactam antibiotics, as penicillins, cephalosporins and aztreonam [13], significantly reducing the therapeutic options and increasing the treatment cost and mortality rates [14-17]. β -lactamases are classified according to the functional group attached [14-15]. They have more than 700 different types of β -lactamases that confer resistance to most β -lactam antibiotics [18]. Molecules of β -lactamases can be divided into three major groups of ESBL enzymes; TEM, SHV and CTX-M, further can be divided into some subgroups (name the subgroups) (<http://www.lahey.org/studies>). Among them, CTX-M is reported to have more than 100 types of enzyme, categorized on the basis of amino acid sequences. CTX-M variants have been divided into 5 subgroups, CTX-M1, CTX-M2, CTX-M8, CTX-M9 and CX-M25 [19,20]. During the last decade, CTX-M enzymes have become the most prevalent ESBL enzymes, especially in ESBL producing *E. coli* in Europe, Asia and South America [21]. The ESBL producing bacterium, harbor a number of genes that encode different virulence factors and causes pathogenicity [22-23]. Plasmid mediated transmission of genes, encoding β -lactamases play a role in resistance of *E. coli*. These genes most often carry resistance determinants for broad spectrum of cephalosporin and making the microorganisms resistant to almost all antibiotics [24], third generation cephalosporin and broad-spectrum antibiotics drug [25-27]. These resistance determinants can be transferred through DNA horizontal transfer of plasmids, bacteriophages and transposons even between species and allow the bacteria to increase the ability to cause a broad spectrum of diseases [28-29]. The characterizations of virulence genes play an important role in improving our understanding of the pathogenesis of UTIs and help to minimize the complications, including kidney failure.

The present study was proposed to determine the frequency of important resistance genes and quantification by real time PCR. The method was able to produce rapid and accurate results with high sensitivity and specificity.

2. MATERIALS AND METHODS

2.1 Sample Collection and Antimicrobial Assay

A total of one hundred fifty urine samples from different UTIs patients with diabetes mellitus were collected from health Center, Imam

University, Riyadh, Kingdom of Saudi Arabia, in sterile plastic universal containers and kept in ice cold conditions by adding 1.8 % boric acid-sodium formate as a bacteriostatic compound [30]. *E. coli* was present in 36% of samples (Table 1). The samples have been collected from both males and females' patients (25-65 age), and taken their medical history and routine life styles. The samples have been cultured for identification of *E. coli*, colonies were selected and characterized on the basis of Gram's staining, their morphology, cultural and biochemical characteristics according to the key of Bergey's Manual of Systematic Bacteriology [31-33]. For further experiments, the cultured samples were stored in cryovials at -20°C after adding Luria-Bertani broth with 30% glycerol (Invirogen, USA)

In regard to multidrug resistance, antimicrobial susceptibility tests were performed for the *E. coli* isolates with Kirby Bauers disc diffusion method on the Mueller-Hinton agar (Sigma, USA) [31], as recommended by the Clinical and Laboratory Standards Institute (CLSI) [32-33]. Different antibiotic disks were tested, as ampicillin (20 μ g), amoxicillin (20 μ g), amikacin (30 μ g), cefotaxime (30 μ g), ciprofloxacin (5 μ g), chloramphenicol (30 μ g), ceftazidime (30 μ g), gentamicin (30 μ g), nitrofurantoin (300 μ g), norfloxacin (10 μ g), tetracycline (30 μ g) and clavulanic acid (10 μ g) (Sigma Aldrich, USA). The isolates with MICs were recorded using the sensitivization system (Trek Diagnostic Systems), values ≤ 1 , and $\geq 4\mu$ g/mL considered as resistant and susceptible, respectively at 37 °C for 24 hours (Table 2). The results were calculated as mean with one standard deviation (SD) [34].

2.2 Screening for ESBL Phenotype

The presence of ESBL phenotype was confirmed by using disc diffusion on Mueller-Hinton agar with combined disc of cefotaxime and cefotaxime-clavulanic acid, ceftazidime and ceftazidime/clavulanic acid at 37°C for 24 hours [31-32]. MIC value of cephalosporin plus-clavulante disc was equal to or greater than 5 mm in diameter of inhibition zones around the disks and compared to the inhibition zones around disks free of clavulanic acid, interpreted as phenotypic confirmation of ESBL production [31]. Combination disks containing ceftazidime and clavulanic acid were placed at a distance of 25 mm from each other. The double-disk synergy test was performed according to established protocols and all experiments were carried out at

least three times. The results were expressed as mean with one standard deviation (SD) [34].

2.3 DNA Extraction

Genomic DNA was extracted from fresh cultured bacteria on agar medium suspended in TE buffer by using a DNeasy kit (Qiagen, Germany) according to the instruction of the manufacturer. The DNA was subjected to polymerase chain reaction (PCR) after determining concentration and quality by using a nano drop spectrophotometer at A_{260} and A_{280} nm absorbance and 0.6% agarose gel electrophoresis, respectively. Freshly extracted DNA was used for PCR or stored at -20°C in Tris-EDTA buffer until analysis.

3. RESISTANCE GENES

3.1 Detection of Resistance Genes by PCR

Extracted DNA was screened by PCR for TEM, SHV and CTX-M genes, the primers sequences of these genes and control gene (house keeping β -actin) designed with reference to the National Centre for Biotechnology Information (NCBI) database for homologous nucleotide sequences by BLAST (Basic Local Alignment Search Tool), are listed in Table 3. PCR reactions were carried out in 50 μl PCR tubes using specific primers and appropriate annealing temperature for amplifying the TEM, SHV and CTX-M genes. Total volume of PCR reaction was 25 μl containing; 2.5 μl of 10X PCR reaction buffer, with MgCl_2 (1.5 mM), 0.5 μl of dNTPs (10 mM), 0.5 μl of each primer (20 pM/ μl), 5U/ μl Taq DNA polymerase (Sigma Aldrich, USA) and 50 ng DNA template. Amplifications were carried out in a thermal cycler (Ependroff, USA), using the following conditions: Initial amplification at 95°C for 2 min; 35 cycles at 94°C for 30 s, 55°C for 50 s, and 72°C for 50 s; final extension at 72°C for 2 min. PCR amplified products were analyzed in 2% agarose gel stained with ethidium bromide at 10V/cm electrophoresis, visualized under ultraviolet (UV) light and photographed using gel documentation system (Image Master VDS, Pharmacia, USA).

3.2 Quantification of Resistance Genes by Real Time PCR (RT-PCR)

After screening of TEM, SHV and CTX-M genes, quantification of these gene by RT-PCR with the same sequenced primers that were used in PCR.

RT-PCR reactions were conducted on Light Cycler 480 II system with software version 1.5 (Roche Diagnostics, Mannheim, Germany) with the SyBr Green I[®], a nucleic acid stain (Invitrogen, USA) according to the manufacturer's instructions. RT-PCR reaction was carried out in 50 μl PCR plate using specific temperatures for quantification of the TEM, SHV and CTX-M genes. Total volume of the reaction was 25 μl containing; 50ng of DNA templates, 2.5 μl of 10X PCR reaction buffer (200 mmol/L Tris, pH 8.4, 500 mmol/L KCl, and 2.5 mmol/L MgCl_2), 0.5 μl of deoxyribonucleotides (dNTPs, 300 $\mu\text{mol/L}$), 0.5 μl of each oligonucleotide (20 pM/ μl), 0.5 U/ μl Taq DNA polymerase (Sigma Aldrich, USA), 1:1000 SyBr Green I[®] nucleic acid stain, 100 $\mu\text{g/ml}$ BSA and 1% glycerol final volume maintained with ddH_2O . Amplification was carried out with following conditions: Initial denaturation at 95°C for 2 minute followed by 40 PCR cycles, 94°C for 20 s, 58°C for 30 s, and 72°C for 50 s. Fluorescence was detected at the end of each annealing step, 58°C segment. Following amplification, a melting curve was generated by heating the PCR product to 95°C with a ramp rate of 0.05°C/s . Polymerase chain reaction products of house keeping β -actin gene were used as a standard. Amplifications were performed in duplicate for the entire samples. Real-time PCR analysis and subsequent calculations were performed on Light Cycler 480 II system with software version 1.5. The relative starting quantity was determined using the threshold cycle of each samples, and analyzed by the $2^{-\Delta\text{Ct}}$ or $2^{-(\text{Ct of target} - \text{Ct of internal control})}$ formula [35].

4. RESULTS

In the present study, 150 urine samples from UTIs with Diabetes Mellitus were collected after concern with patients and as per ethical guidelines for the culture and isolates were identified by using conventional cultural, biochemical and morphological tests (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. mirabilis*, *P. vulgaris* and *S. aureus*) (Table 1) 36% (54/150) of *E. coli* isolates were recovered. Out of total, numbers of the female samples were 86.66% (130/150) and males' samples were 13.33% (20/150). Among these strains, positive strains revealed the ESBL phenotype by using clavulanic acid as a β -lactamase inhibitor in the phenotypic confirmatory disk diffusion test. All the isolates were tested for prevalence of resistance for twelve different antibiotics ampicillin, amoxicillin, amikacin, cefotaxime,

ciprofloxacin, chloramphenicol, gentamicin, nitrofurantoin, norfloxacin, tetracycline, ceftazidime and amoxicillin/clavulanic acid. Antibiotic resistance rates were observed in different pattern in non ESBL and ESBL producing *E. coli*. In non ESBL *E. coli* the following antibiotics pattern were observed, ampicillin 72.72%, amoxicillin 66.66%, amikacin 39.39%, cefotaxime 33.33%, ceftazidime 12.12%, chloramphenicol 39.39%, ciprofloxacin 15.15%, gentamicin 9.09%, nitrofurantoin 21.21%, norfloxacin 18.18% and tetracycline 51.51%. But in ESBL producing the antibiotic resistance rates were found in different pattern as, ampicillin 100%, amoxicillin 100%, cefotaxime 61.90%, tetracycline 95.23%, amikacin 61.90%, nitrofurantoin 66.66%, gentamicin 76.19%, ciprofloxacin 85.71%, chloramphenicol 95.23%, norfloxacin 71.42%, ceftazidime 66.66 and amoxicillin/clavulanic acid 0% (Fig. 1, Table 4). ESBL productions were found to be 35% by using clavulanic acid as a β lactamase inhibitor in the phenotypic. The antibiotics resistance was observed in different patterns, ampicillin and amoxicillin had the highest resistance rate but amoxicillin/clavulanic acid was most effective. Among these strains, 54 (36%) strains revealed the ESBL phenotype by using clavulanic acid as a β -lactamase inhibitor in the phenotypic confirmatory disk diffusion test. ESBL producing isolates possess a higher degree of resistance towards antibiotics as compared to non ESBL producing isolates.

All *E. coli* isolates were screened for the presence of commonly detected uropathogenic *E. coli* virulence genes TEM, SHV and CTX-M genes. Relationship between the distribution of virulence genes and resistance to multiple drugs

was also investigated. The ESBL encoding genes were characterized by PCR on overall phenotypic results obtain with ESBL tests. The specific primers were used on the isolated DNA for ESBL producing isolates for the characterization and quantification of TEM, SHV and CTX-M genes. The positive PCR products of the CTX-M, TEM and SHV were obtained in different sizes respectively (Fig. 2). All of the genes, preliminary identified in conventional PCR formats, were detected on the real time PCR and none of the negative isolates turned out to be positive. Among these isolates, CTX-M was significantly higher 85.4% in the ESBL positive strains ($p < 0.05$). TEM and SHV were found 12.8% and 6.5%, respectively (Fig. 3). The negative controls for the establishment of the real-time PCR assay used negative isolates in conventional PCR and after completion of the experiment, a cycle threshold (C_t) was calculated by determining the signal strength at which the fluorescence exceeded a threshold limit. No amplification was obtained in negative samples but in positive samples the threshold, line cycle threshold (C_t) values showed the variability. Furthermore, no false positive fluorescence was detected. Samples possessing a fluorescence signal above a specific value were assessed as positive. The fluorescence curves exceeded the cycle threshold after completion of around 50% of real time run, between cycles. The highest standard deviation C_t value was 4.6 for TEM gene, for CTX-M and SHV, 2.4 and 1.5 respectively (Fig. 4). Some quantification differences observed, TEM gene found more in quantity than other genes SHV and CTX-M. All samples were analyzed in duplicates using the same settings.

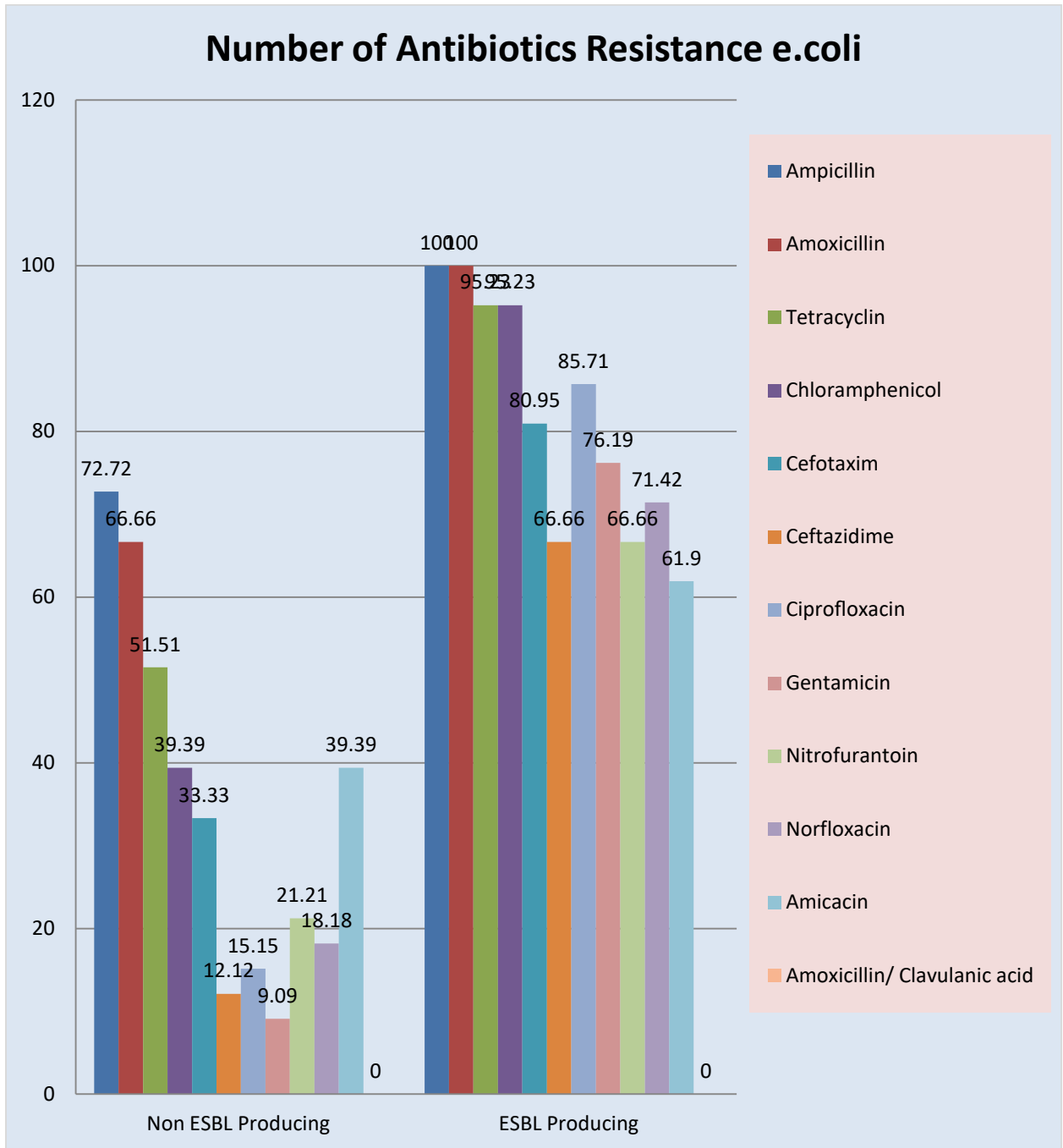


Fig. 1. Antibiotic resistance and suitability rates in non-ESBL and ESBL producing e. coli

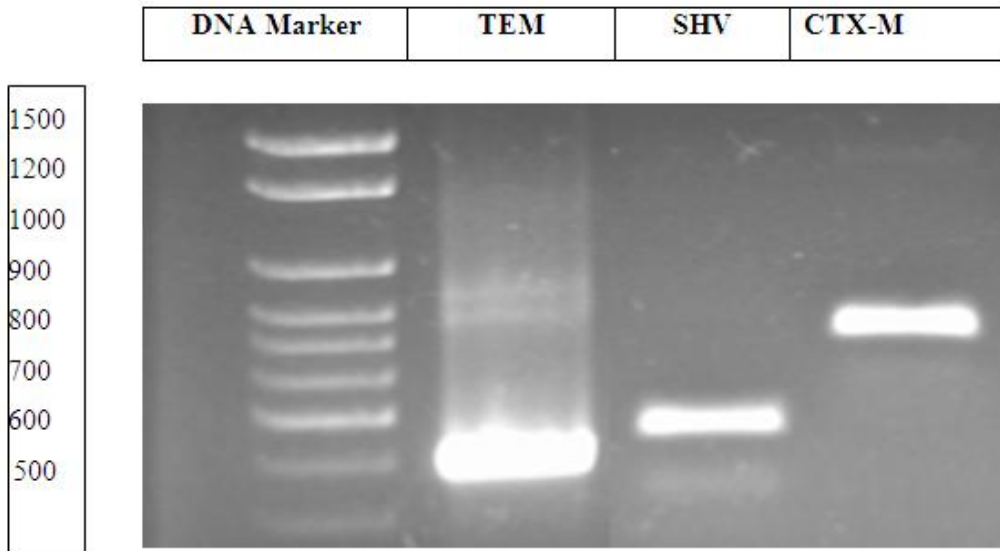


Fig. 2. PCR amplicon of CTX-M, TEM and SHV genes, respectively

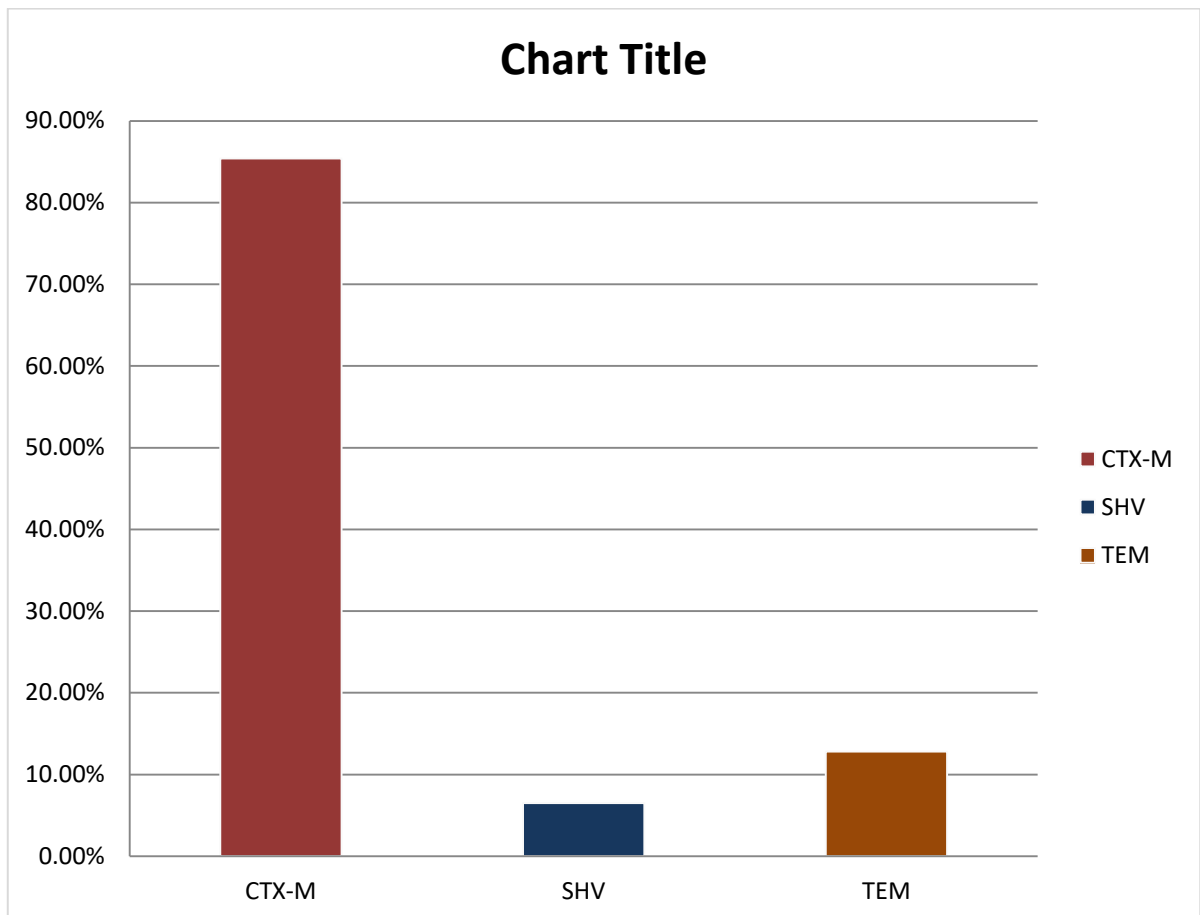


Fig. 3. Quantification of CTX-M, TEM and SHV genes by Real Time PCR

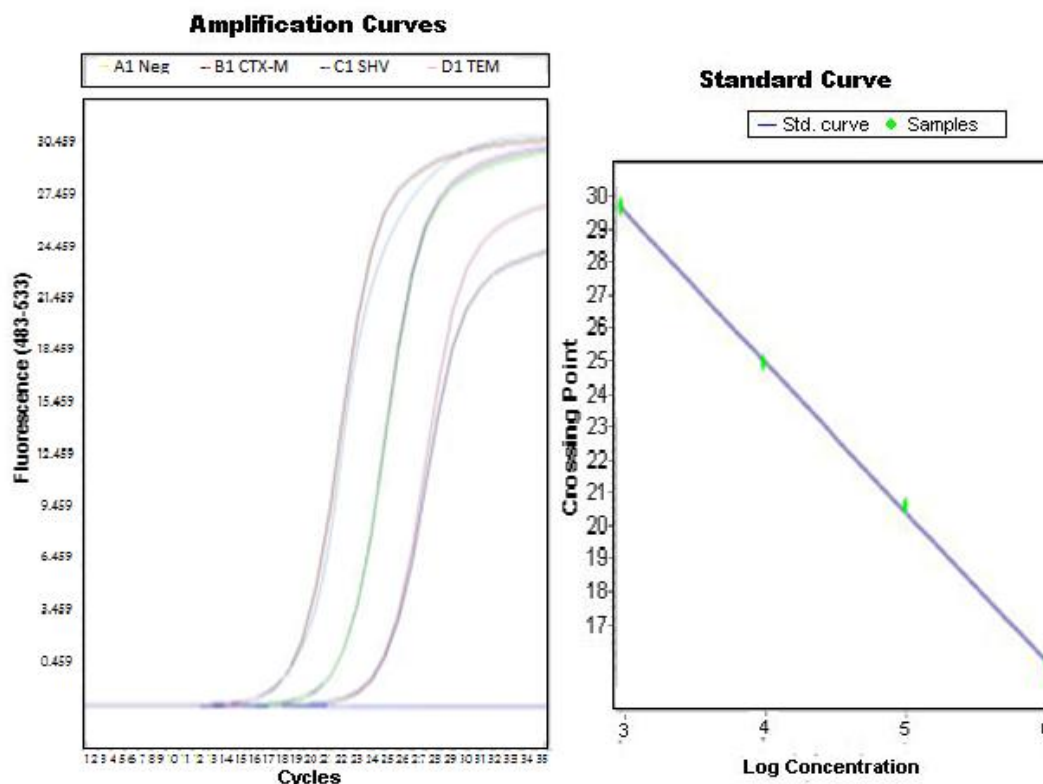


Fig. 4. The fluorescence curves and standard deviation C_t value of CTX-M, TEM and SHV genes respectively, by real time PCR amplifications

Table 1. Number of microbes in collected one hundred fifty urine samples

S. No.	Microbes	No. of positive cases	Percentage (%)
1.	<i>Escherichia coli</i>	54	36.00
2.	<i>Klebsiella pneumoniae</i>	32	21.33
3.	<i>Pseudomonas aeruginos</i>	21	14.00
4.	<i>Proteus vulgaris</i>	20	13.33
5.	<i>Staphylococcus aureus</i>	16	10.66
6.	<i>Proteus mirabilis</i>	7	04.66

Table 2. List of the antibiotics and quantity has to be used

S. No.	Antibiotics	Quantity
1.	Ampicillin	20 µg
2.	Amoxicillin	20 µg
3.	Amikacin	30 µg
4.	Cefotaxime	30 µg
5.	Ceftazidime	30 µg
6.	Chloramphenicol	30 µg
7.	Ciprofloxacin	5 µg
8.	Gentamicin	30 µg
9.	Nitrofurantoin	300 µg
10.	Norfloxacin	10 µg
11.	Tetracycline	30 µg
12.	Clavulanic acid	10 µg

Table 3. List of primers used for amplification of the TEM, SHV and CTX-M genes

	Sequences (5'-3')	Product size (bp)	References
TEM	CAT TTC CGT GTC GCC CTT ATTC CGT TCA TCC ATA GTT GCC TGAC	800	32
SHV	AGC CGC TTG AGC AAA TTA AAC ATC CCG CAG ATA AAT CAC CAC	713	32
CTX-M	TTA GGA AGT GTG CCG CTGA CGA TAT CGT TGG TGG TCCT	688	32

Table 4. Antibiotics resistance and suitability rate in non-ESBL and ESBL producing *e. coli*

S. No.	Drug	<i>E. coli</i> Isolates (n=54)	
		Non ESBL Producing (61.11%)	ESBL Producing (38.88%)
1.	Ampicillin	24 (72.72%)	21 (100%)
2.	Amoxicillin	22 (66.66%)	21 (100%)
3.	Amikacin	13 (39.39%)	13 (61.90%)
4.	Cefotaxime	11 (33.33%)	17 (80.95%)
5.	Ceftazidime	4 (12.12%)	14 (66.66%)
6.	Chloramphenicol	13 (39.39%)	20 (95.23%)
7.	Ciprofloxacin	5 (15.15%)	18 (85.71%)
8.	Gentamicin	3 (9.09%)	16 (76.19%)
9.	Nitrofurantoin	7 (21.21%)	14 (66.66%)
10.	Norfloxacin	6 (18.18%)	15 (71.42%)
11.	Tetracycline	17 (51.51%)	20 (95.23%)
12.	Amoxicillin/ Clavulanic acid	0 (0%)	0 (0%)

5. DISCUSSION

Urinary tract infections are more common particularly in women and severe often caused by antibiotic resistance and carry worse consequences. Patients undergoing pharmacologic treatment for diabetes were mainly at higher risk of UTIs and severity of diabetes. The higher risk of UTIs with longer duration in diabetic patients may cause diabetes complication such as neuropathy of retinopathy [36]. The prevalence of bacteriuria increased 1.9-fold every 10 years of diabetes duration [37]. This is probably due to higher prevalence of autonomic neuropathy and subsequent incomplete bladder emptying in longstanding diabetes [38]. Various studies have estimated the prevalence of UTIs among patients with diabetes. One study obtained 12.0% prevalence of UTIs in diabetic patients and prevalence in females (17.7%) significantly higher than in males (5.2%), similar results as in United States [39-40]. Our results are in concordance with Saudi Arabian and Indian population where the prevalence of UTIs in diabetes is higher (41.1%)

in females than males (7.2%) [39]. We also observed that *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae*, *E. coli*, *P. vulgaris* and *S. aureus* were predominant microorganisms present in UTIs (Table 1). In last few years, a significant increase in the number of ESBL associated *E. coli* has been reported in several parts of the world [41-42]. A frequent irrational use of antibiotics changes the intestinal flora, leading to bacterial resistance [38].

ESBL associated *E. coli* has been considered a serious public health/infection control issue particularly in intensive care units and in severely immunocompromised patients and have been associated with increased morbidity and mortality [43-45]. β -lactamases enzymes are encoded on larger sized plasmids, especially due to plasmid mediated transmission of the encoding genes. According to a study, 90% uropathogenic *E. coli* showed at least one of the eight virulence genes, conducted by Oliveira et al. [46]. ESBL genes have been reported as CTX-M (CTX-M-15, CTX-M-14, CTX-M-12, CTX-M3), TEM (1, 2) PSE, and OXA.TEM, SHV and CTX-M are the most

common types, widespread and frequently detected in *E. coli* [47]. A great diversity of genes associations indicating heterogeneity in distribution of virulence genes among uropathogenic *E. coli* strains in different regions [46].

Recently, many studies have reported ESBL producing *E. coli*. A recent report from Cameroon showed 16% fecal carriage of ESBL isolates with the majority of these being *E. coli* and 95.6% from Saudi Arabia and 4.4% were *k. pneumoniae* out of 12.7% [48]. In Switzerland presence was 64% in the community and 36% in healthcare associated UTIs [49]. Spain had presence of 68% in the community and 32% in healthcare associated [50]. The frequency of O25b-ST131 was 24.7% in Iran in 2017 [51]. The presence of O25b-ST131 clone in the strains isolated, had been reported as 22.8% and 55.6% respectively from outpatients and inpatients. In ESBL positive, the presence of O25b-ST131, CTX-M-1 and CTX-M-15 were reported 78.5%, 100%, 95.5%, respectively and ESBL presence in *E. coli* strain were 47% in 2016 in Indian [52]. Taval et al., found *E. coli* strains as causative agents in 265 patients with UTIs in Germany in 2014 [53]. The incidence of ESBL producing strains has been reported to be 68% (69 of 101) in China and 40% (60 of 150) in United States and the highest prevalence of ESBL has been reported in ICU patients in Japan [54]. A study from France in 2016, reported eleven ESBL positive *E. coli* strains out of which found CTX-M15 in 5 and CTX-M1 in 4, respectively [55]. In Istanbul CTX-M15 gene was 14% and O25b-ST131 clone 12% by Can et al., [56].

In our study, we focused on the *E. coli* strains isolated from patients with UTIs and their sensitivity patterns to different commonly used antibiotics. It was observed that the prevalence of ESBL in *E. coli* isolates was 38.88 % (21/54). The highest percentage of resistance rate in non ESBL producing isolates were 72.72 % and 100% in ESBL producing isolates. Ampicillin and amoxicillin showed 100% resistance in ESBL producing isolates but in non ESBL producing isolates 72.72% and 66.66%, respectively. Many ESBL genes have been reported but TEM, SHV and CTX-M, are the most widespread and frequently detected genes in *E. coli*. We focused on three types of ESBL genes in this study i.e. CTX-M, SHV and TEM. CTM-X gene has a high occurrence than TEM and SHV genes; CTX-M was found 85.4 %, TEM (12.8%) and SHV (6.5%) respectively. The findings are in

accordance with previous literature, where CTX-M genes were found to be quite high in clinical isolates in a study conducted in France and Chinese urban populations [49]. Most of the ESBL producers had CTX-M gene. Genotyping of ESBL encoding genes revealed that β -lactamase families are associated with high spectrum activity against β -lactamases [57]. Molecular identification of β -lactamases could be essential for a reliable epidemiological investigation of antimicrobial resistance.

6. CONCLUSION

In this study we found that UTIs is the more common disease in diabetic patients and *E. coli* is the most frequent microorganism, ESBL producing *E. coli* were the most antibiotic resistant pathogen in UTI and treatment without proper diagnosis may lead to antimicrobial drug resistance. Genotyping of ESBL producing genes indicated that β -lactamase families associated with high spectrum activity against β -lactamases and found three more common types of ESBL genes: CTX-M, SHV and TEM. Early and proper diagnosis is necessary for management, helping to prevent the inappropriate use of antibiotics, reduce the morbidity and mortality of diabetic patients suffering from UTIs.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

As per international standard or university standard, Participants' written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The procedures involving patients' participants were conducted in accordance with the ethical standards of the responsible institutional research committee and author have all ethical clearance related to the study, regulation of the Imam Mohammad Ibn Saud Islamic University,

Government of Saudi Arabia. (IRB no. 00112/10/2016).

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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