Evaluation and quantification of resistant gene in antibiotic resistant ESBL producing *E. coli* in UTI samples with diabetes mellitus: A Molecular Study

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.

Key words: 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 coat 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 (20µg), amoxicillin (20µg), amikacin (30µg), cefotaxime (30µg), ciprofloxacin (5µg), chloramphenicol (30µg), ceftazidime (30µg), gentamicin

(30µg), nitrofurantoin (300µg), norfloxacin (10µg), tetracycline (30µg) and clavulanic acid (10µg) (Sigma Aldrich, USA). The isolates with MICs were recorded using the sensititrevizion system (Trek Diagnostic Systems), values ≤ 1, and ≥ 4µg/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 ceftazidine/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 50µl 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 50µl PCR plate using

specific temperatures for quantification of the TEM, SHV and CTX-M genes. Total volume of the reaction was 25µl containing; 50ng of DNA templates, 2.5µl of 10X PCR reaction buffer (200 mmol/L Tris, pH 8.4, 500 mmol/L KCl, and 2.5 mmol/L MgCl2), 0.5 µl of deoxyribonucleotides (dNTPs,300 µmol/L), 0.5µl 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 µg/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 $2^{-\Delta Ct}$ 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%, ceftazidine 12.12%, chloramphenicol 39.39%, ciprofloxacin 15.15%, gentamicin 9.09%, nitrofurantoin 21.21%, norfloxacin 18.18% and tetracyline 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% (Figure 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 (Figure 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 (Figure 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 (Figure 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.

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 in 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. pneumonia* 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.

CONCLUTION

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.

ETHICAL APPROVAL

Institutional Review Board Statement: 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).

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. 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.

Acknowledgement: The Authors also extend their appreciation to the Deanship of Scientific Research at Imam Mohammad Ibn Saud Islamic University for funding through Research Group No. RG-21-09-93.

REFERENCESR

1. Boyko EJ, Fihn SD, Scholes D. Risk of urinary tract infection and asymptomatic becteriuria among diabetic and nondiabetic postmenopausal woman. Am J Epidemio. 2005; 161: 557.
2. Calbo E, Romani V, Xercavins M, Gomez L, Vidal CG, Quintana S. Risk factors for community-onset urinary tract infections due to *Escherichia coli* harbouring extended-spectrum beta-lactamases. J Antimicrob Chemother. 2006; 57:780-3.
3. Topaloglu R, Er I, Dogan BG, Bilginer Y, Ozaltin F, Besbas N. Risk factors in community-acquired urinary tract infections caused by ESBL-producing bacteria in children. Pediatr Nephrol. 2010; 25:919-25.
4. Fan NC, Chen HH, Chen CL, Ou LS, Lin TY, Tsai M.H. Rise of community-onset urinary tract infection caused by extended-spectrum beta-lactamase-producing *Escherichia coli* in children. J Microbiol Immunol Infect. 2014; 47:399-405.

5. Dayan N, Dabbah H, Weissman I, Aga I, Even L, Glikman D. Urinary tract infections caused by community-acquired extended-spectrum beta-lactamase-producing and nonproducing bacteria: a comparative study. J Pediatr. 2013; 163:1417-21.
6. Marcus N, Ashkenazi S, Yaari A, Samra Z, Livni G. Non-*Escherichia coli* versus *Escherichia coli* community-acquired urinary tract infections in children hospitalized in a tertiary center: relative frequency, risk factors, antimicrobial resistance and outcome. Pedia Inf Dis J 2005; 24(7):581-5.
7. Schappert, SM. Ambulatory care visits to physician offices, hospital outpatient departments, and emergency departments: United States, 1997. National Center for Health Statistics. Vital Health Stat. 1999; 13(143):1-39.
8. Foxman B. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am J Med. 2002; 113:S5–S13.
9. Chanoine MHN, Bertrand X, Madec JY. *Escherichia coli* ST131, an intriguing clonal group. Clin Microbiol Rev. 2014; 27:543-574.
10. Lee JH, Subhadra B, Son YJ, Kim DH, Park HS, Kim JM. Phylogenetic group distributions, virulence factors and antimicrobial resistance properties of uropathogenic *Escherichia coli* strains isolated from patients with urinary tract infections in South Korea. Lett Appl Microbiol. 2016; 62:84-90.
11. Walker KE, Mahon CR, Lehman D, Manuselis. Enterobacteriaceae G. In: Mahon CR, Lehman D, Manuselis G, editors. Textbook of diagnostic microbiology. 5th ed. New York: W. B. Saunders Company 2015:420-54.
12. Wayne PA. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing 25th informational supplement 2015:M100-S25.

13. Khoshnood S, Heidary M, Mirnejad R. Drug-resistant gram-negative uropathogens: a review. Biomed Pharmacother. 2017; 94:982-994.
14. Bano RJ, Alcala JC, Cisneros JM, Grill F, Oliver A, Horcajada JP. Community infections caused by extended-spectrum beta-lactamase-producing *Escherichia coli*. Arch. Intern. Med. 2008; 168:1897-1902.
15. Ami BR, Bano RJ, Arslan H, Pitout JD, Quentin C, Calbo ES. A multinational survey of risk factors for infection with extended-spectrum beta-lactamase-producing Enterobacteriaceae in nonhospitalized patients. Clin Infect Dis. 2009; 49:682-690.
16. Yilmaz E, Akalin H, Ozbey S, Kordan Y, Sinirtaş M, Gurcuoglu E. Risk factors in community-acquired/onset urinary tract infections due to extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae*. J Chemother 2008; 20:581-585.
17. Azap OK, Arslan H, Serefhanoglu K, Colakoglu S, Erdogan H, Timurkaynak F. Risk factors for extended-spectrum beta-lactamase positivity in uropathogenic *Escherichia coli* isolated from community-acquired urinary tract infections. Clin Microbiol Infect. 2010; 16:147-151.
18. Bush K, Jacoby GA. Updated functional classification of *β*-lactamases. Anti Agents and Chemo. 2010; 54:969-976.
19. Peirano G, Pitout JDD. Molecular epidemiology of *Escherichia coli* producing CTX-M beta-lactamases: the world-wide emergence of clone ST131 O25:H4. Inter J Anti Agents. 2010; 35(4):316-321.
20. Canton R, Coque TM. The CTX-M *β*-lactamase pandemic. Current Opinion Micro. 2006; 9(5):466-475.

21. Bonnet R. Growing group of extended-spectrum *β*-lactamase: the CTX-M enzymes. Antimicro. Agents Chemother. 2004; 48:1-14.
22. Anago E, Ayi-Fanou L, Akpovi CD, Hounkpe WB, Tchibozo ADM, Bankole HS. Antibiotic resistance and genotype of beta-lactamase producing Escherichia coli in nosocomial infections in Cotonou, Benin. Ann Clin Microbiol Antimicro. 2015; 14:5.
23. Ahoyo AT, Baba-Moussa L, Anago AE, Avogbe P, Missihoun TD, Loko F. Incidence of infections dues to Escherichia coli strains producing extended spectrum betalactamase, in the Zou/Collines Hospital Centre (CHDZ/C) in Benin. Med Mal Infect. 2007; 37(11): 746-52.
24. Accogli MT, Giani M. Emergence of Escherichia coli ST131 sub-clone H30 producing VIM-1 and KPC-3 carbapenemases Italy. J Anti Chemoth. 2014; 69(8):2293-2296.
25. Cai JC, Zhang R, Hu YY, Zhou HW, Chen GX. Emergence of Escherichia coli sequence type 131 isolates producing KPC-2 carbapenemase in China. Antimicro Agents Chemoth. 2014; 58(2):1146-1152.
26. Johnson TJ, Hargreaves M, Shaw K. Complete genome sequence of a carbapenem-resistant extraintestinal pathogenic *Escherichia coli* strain belonging to the sequence type 131 H30R subclade. Genome Announcements. 2015; 3(2):1-2.
27. Rogers BA, Sidjab HE, Paterson DL. Escherichia coli O25b-ST131: a pandemic, multi resistant, community associated strain. J Antimicro Chemoth. 2011; 66 (1):1-14.
28. Bush K, Jacoby GA. Updated functional classification of *β*-lactamases. Anti Agents Chemoth. 2010; 54(3):969-976.

29. Roy C, Foz A, Segura C, Tirado M, Foster C, Reig R. Plasmid-determined β-lactamases identified in a group of 204 ampicillin-resistant Enterobacteriaceae. J Antimicro Chemoth. 1983; 12(5):507-510.
30. Shameli K, Ahmad MB, Zargar M, Yunus WM, Ibrahim NA, Shabanzadeh P, Moghaddam MG. Synthesis and characterization of silver/ montmorillonite/ chitosan bio nano composites by chemical reduction method and their antibacterial activity. Int J Nanomed. 2011; 6:271-284.
31. Brady C, Cleenwerck I, Venter S, Coutinho T, Vos PD. Taxonomic evaluation of the genus Enterobacter based on multilocus sequence analysis (MLSA): proposal to reclassify, respectively, and emended description of the genera Enterobacter and Cronobacter. Syst Appl Microbiol. 2013; 36: 309-319.
32. Grimont PAD, Grimont F. Genus: Klebsiella, In: Volume Two: The Proteobacteria , Part B: The Gammaproteobacteria. In: Brenner DJ, Krieg NR, Staley JT (Edn.) Bergey's Manual of Systematic Bacteriology, (2nd edn), Springer, New York 2005; 685-693.
33. Fazzolari E, Mariotti A, Germon JC. Nitrate reduction to ammonia: A dissimilatory process in Enterobacteramnig enus. Can J Microbiol. 1990; 36:779-785.
34. Altman, Douglas G, Bland JM. Standard deviations and standard errors. BMJ. 2005; 331(7521): 903.
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 ((-Delta Delta C (T)) method. Methods. 2001; 25:402-8.
36. Harris M, Cowie C, Stern M, Boyko E, Reiber G, Bennett P, Bethesda MD. Summary. In Diabetes in America. 2nd ed. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases: 1995; 1-13.

37. Bahl AL, Chugh RN, Sharma KB. Asymptomatic bacteremia in diabetics attending a diabetic clinic. Indian J Med Sci. 1970; 24:1-6.

38. Keane EM, Boyko EJ, Reller LB. Hamman RF. Prevalence of asymptomatic bacteriuria in subjects with NIDDM in San Luis Valley of Colorado. Diabetes Care. 1988; 11:708-12.

39. Boyko EJ, Fihn SD, Scholes D, Abraham L, Monsey B. Risk of urinary tract infection and asymptomatic bacteriuria among diabetic and non-diabetic postmenopausal women. Am J Epidemiol. 2005; 161(6):557-564.

40. Grandy S, Fox KM, Hardy E. Prevalence and recurrence of urinary tract and genital infections among adults with and without type 2 diabetes mellitus in the general population: a longitudinal cohort study. J Diabetes Res Clin Metab 2013; 2(5):8.

41. Cizman M. The use and resistance to antibiotics in the community. Int J Antimicro Agents. 2003; 21(4): 297-307.

42. Pitout JDD, Sanders CC, Sanders WE. Antimicrobial resistance with focus on β-lactam resistance in gram- negative bacilli. Am J Medicine. 1997; 103(1):51-59.

43. Walker KJ, Lee YR, Klar AR. Clinical Outcomes of Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae Infections with Susceptibilities among Levofloxacin, Cefepime, and Carbapenems. Can J Infect Dis Med Microbiol. 2018; 2018: 3747521.

44. Walker E, Lyman A, Gupta K, Mahoney MV, Snyder GM, Hirsch EB. Clinical Management of an Increasing Threat: Outpatient Urinary Tract Infections Due to Multidrug-Resistant Uropathogens. Clin Infect Dis. 2016; 63:960-965.

45. 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*. J Microbiol Immunol Infect. 2010; 43:240-248.
46. Oliveira FA, Paludo KS, Arend LN, Farah SM, Pedrosa FO, Souza EM. Virulence characteristics and antimicrobial susceptibility of uropathogenic *Escherichia coli* strains. Genet Mol Res. 2011; 10:4114-25.
47. Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. Lancet Infect Dis. 2008; 8(3):159-166.
48. Niki M, Hirai I, Yoshinaga A. Extended-Spectrum *β*-lactamases (ESBLs)-producing *Escherichia coli* strains in the feces of carriers contribute substantially to urinary tract infections in these patients. Infection 2011; 39(5):467-471.
49. Meier S, Weber R, Zbinden R, Ruef C, Hasse B. Extended-spectrum *β*-lactamase-producing Gram-negative pathogens in community-acquired urinary tract infections: an increasing challenge for antimicrobial therapy. Infection 2011; 39(4): 333-340.
50. Bano JR, Alcala JC, Cisneros JM. Community infections caused by extended-spectrum *β*-lactamases (ESBLs)-producing *Escherichia coli*. Archives Inter Medicine. 2008; 168(17): 1897-1902.
51. Namaei MH, Yousefi M, Ziaee M, Salehabadi A, Ghannadkafi M, Amini E. First report of prevalence of CTX-M-15-producing *Escherichia coli* O25b/ST131 from Iran. Microb Drug Resist. 2017; 23:879-884.

52. Marialouis XA, Santhanam A. Antibiotic resistance, RAPD- PCR typing of multiple drug resistant strains of *Escherichia Coli* from urinary tract infection (UTI). J Clin Diagn Res. 2016; 10: 5-9.

53. Toval F, Kohler CD, Vogel U, Wagenlehner F, Mellmann A, Fruth A. Characterization of *Escherichia coli* isolates from hospital inpatients or outpatients with urinary tract infection. J Clin Microbiol. 2014; 52: 407-418.

54. Hayami H, Takahashi S, Ishikawa K. Nationwide surveillance of bacterial pathogens from patients with acute uncomplicated cystitis conducted by the Japanese surveillance committee during 2009 and 2010: antimicrobial susceptibility of *Escherichia coli* and *Staphylococcus saprophyticus*. J Infection Chemoth. 2013; 19(3): 393-403.

55. Rabenirina RG, Malespine J, Ducat C, Sadikalay S, Falord M, Harrois D. Temporal trends and risks factors for antimicrobial resistant Enterobacteriaceae urinary isolates from outpatients in Guadeloupe. BMC Microbiol. 2016; 16:121.

56. Can F, Azap OK, Seref C, Ispir P, Arslan HO. Ergonul Emerging *Escherichia coli* O25b/ST131 clone predicts treatment failure in urinary tract infections. Clin Infect Dis. 2015; 60:523-527.

57. Chaudhary AA, Fareed M, Malik A, Khamees OA. Molecular Characterization of Resistance to ESBL Producing *E. coli* and Development of LAMP Assay against the Microbes for UTIs Patients. Int J Med Res Health Sci. 2018; 7(9):112-119.

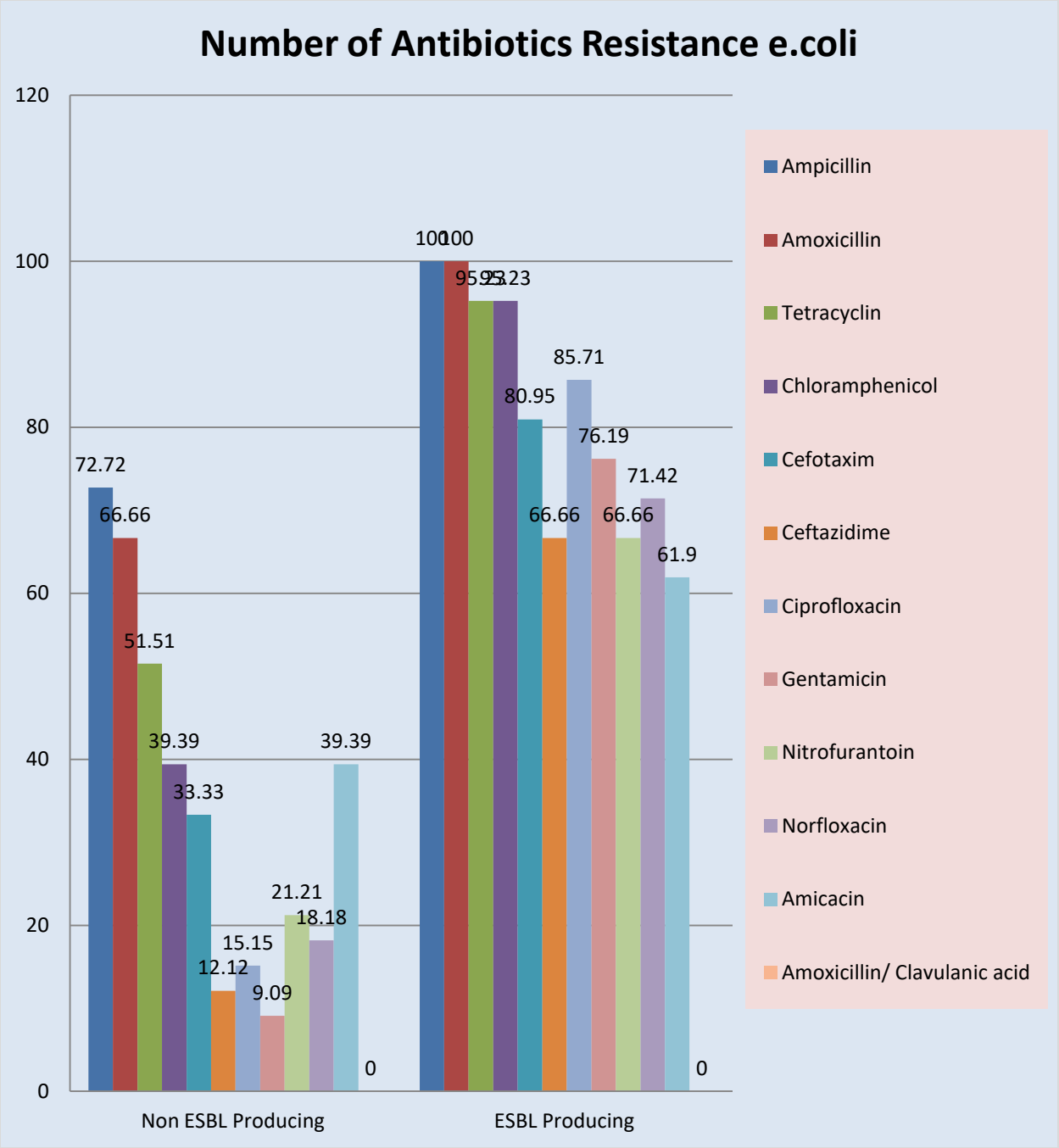

Figure 1. Antibiotic resistance and suitability rates in non-ESBL and ESBL producing *e. coli*.

DNA Marker	TEM	SHV	CTX-M

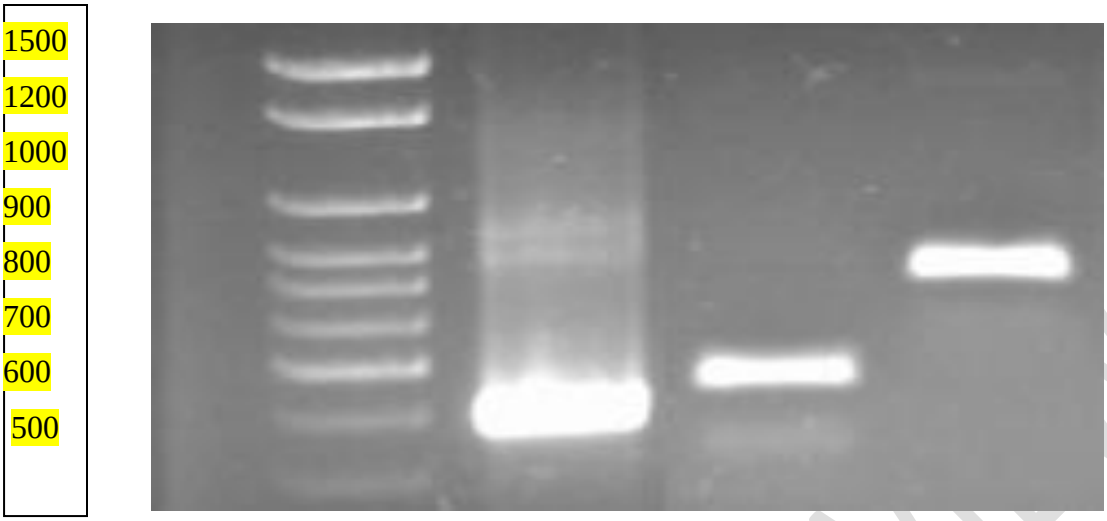

Figure 2. PCR amplicon of CTX-M, TEM and SHV genes, respectively.

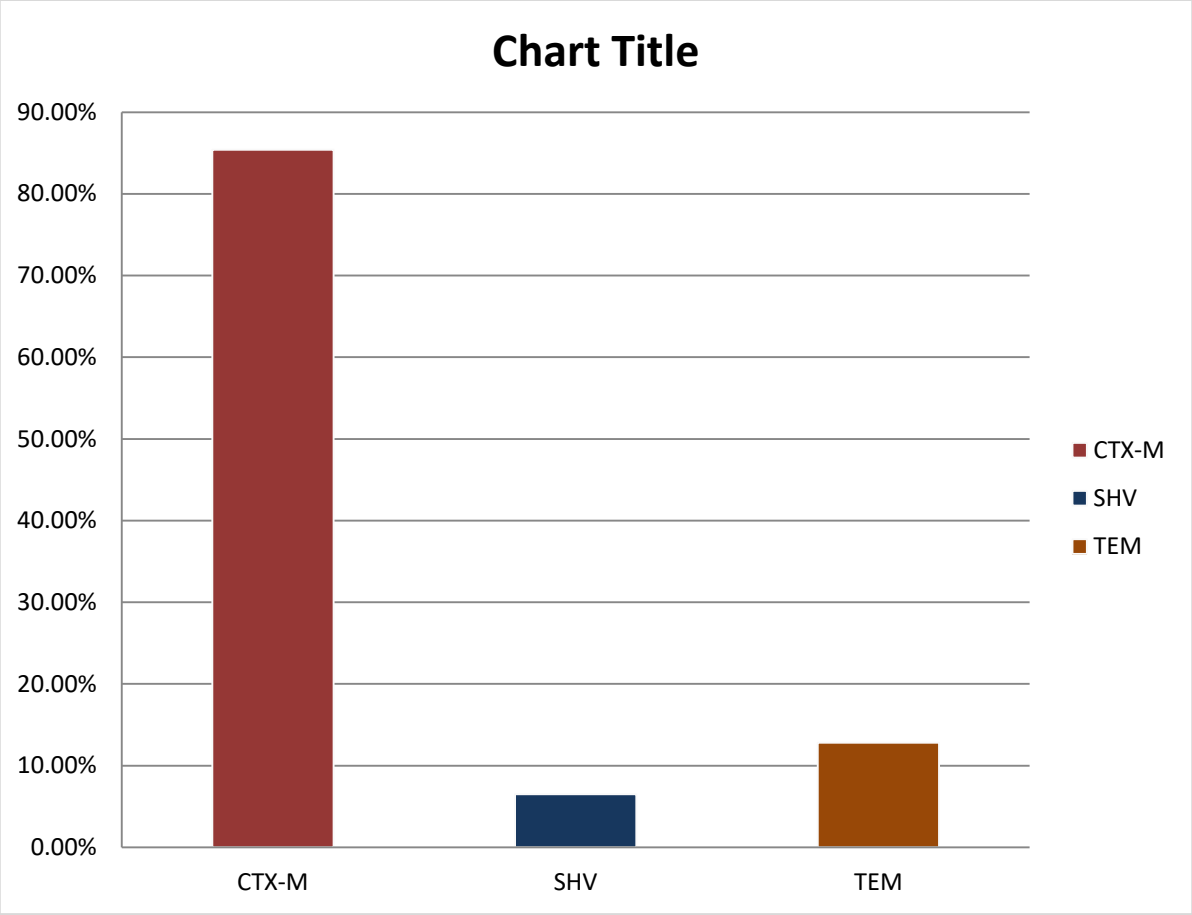

Figure 3. Quantification of CTX-M, TEM and SHV genes by Real Time PCR.

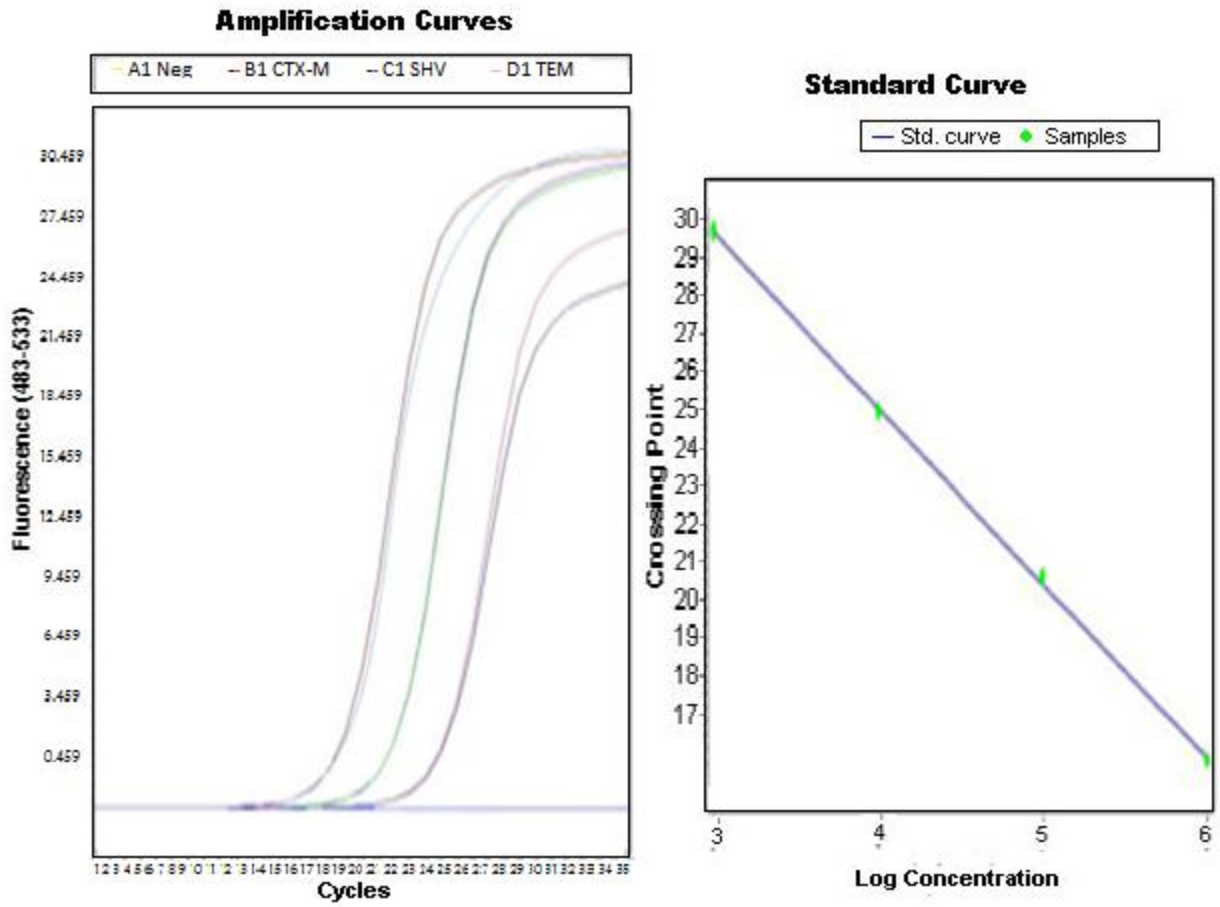

Figure 4. The fluorescence curves and standard deviation C_t value of CTX-M, TEM and SHV genes respectively, by real time PCR amplifications.

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

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