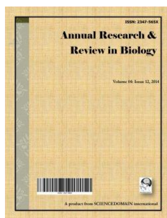

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Phylogeny and Detection of bla_{TEM}, bla_{SHV}, bla_{CTX-M} Genes in *Escherichia coli* Isolates from Patients with Urinary Tract Infections in Taif Hospitals, Saudi Arabia

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Authors' contributions

This work was carried out in collaboration between all authors. Author MMH designed the study, wrote the protocol, helped in molecular part of work and wrote the first draft of the manuscript. Author AG wrote the protocol, cultured the samples and follow up E. coli culture. Author WFA wrote the protocol, worked with the molecular identification, managed the analysis of study. Author EIH helped in manage the analyses of study and managed the literature searches. Author AAM designed the study and managed the literature searches Author AAA helped in collection samples Author AMI helped in follow up E. coli culture. All authors read and approved the final manuscript.

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ABSTRACT

Background: Urinary tract infections (UTIs) represent one of the most common diseases that are encountered in clinical practice and are caused mainly by *Escherichia coli* (*E. coli*).
Aims: The objectives of this study were to identify and compare the bla_{TEM}, bla_{SHV} and $blaC_{TX\text{-}M}$ as marker of beta-lactamase genes in *E. coli* strains isolated from patients with UTIs collecting from King Abdul-Aziz hospital in Taif region, Saudi Arabia.
Study Design: *In vitro* experimental and molecular study.
Place and Duration of Study: Genetic engineering and biotechnology unit, Taif University, from September, 2016 to November, 2017.
Methodology: Beta-lactame antibiotics are prescribed in most infectious disease including UTIs. Twenty one isolates identified as *E. coli* using microbial identification and confirmed by 16S rDNA.
Results: These isolates were susceptible to Imipenem (100%), Ampicillin (90%) and Cefoxitin, but resistant to Cefepime (38%). Existance of selected *bla*-genes (bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$) were detected in the 21 isolates by PCR. Moreover, phylogeny tree was drawn based on 16S rDNA sequence. The results of this study show significant differences in susceptibility to different beta-lactam antibiotics among the *bla-genes* in *E. coli* isolates.
Conclusion: Therefore, our findings instead of our data provide some new epidemiological information about the clonal nature of *E. coli* isolated from patients with UTIs in Taif region, KSA.

Keywords: blaTEM; blaSHV and blaCTX-M genes; 16S rDNA gene; antibiotics; UTI; Escherichia coli.

1. INTRODUCTION

Broad-spectrum beta-lactam antibiotics have commonly been used for treatment of Gram-negative infections. However, bacterial resistance has emerged due to production of extended-spectrum beta-lactamases (ESBLs) [1]. These enzymes are capable of hydrolyzing broad-spectrum beta-lactam antibiotics. ESBLs are derived from genes for the narrow- spectrum beta-lactamases (TEM-1, TEM-2, or SHV-1) by mutations that alter the amino acid configuration around the enzyme active site. They are typically encoded by plasmids that can be exchanged readily between bacterial species [2]. These enzymes are most commonly produced by the members of the *Enterobacteriaceae,* especially *Escherichia coli* and *Klebsiella* [2]. To date, more than 350 different natural ESBL variants are known that have been classified into nine distinct structural and evolutionary families based upon their amino acid sequence comparisons such as TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA, and OXA [1]. The Gram-negative bacterium *Escherichia coli* (*E. coli*) is a common intestinal microorganism of humans and animals, with the capability of acquiring and preserving transferable resistance genes found in other organisms and the environment [2]. In addition, *E. coli* is also an important pathogen causing a variety of illnesses, including Urinary tract infections (UTIs). Beta-lactamases are enzymes that are main cause of bacterial resistance to the beta-lactam antibiotics such as penicillin, cephamycins, carbapenems and cephalosporins family. These enzymes catalyze the hydrolysis of the amide bond of four-membered beta- lactam ring and render the antibiotic inactive against the cell wall transpeptidase, its original cellular target. Based on primary structure, beta-lactamases are grouped into four classes A, B, C, and D enzymes [3]. Beta-lactam antibiotics such as broad spectrum cephalosporins and carbapenems are a good choice in treatment of enterobacterial infections [4,5]. The genes of extended spectrum beta-lactamases (ESBLs) are encoded by transferable plasmids [6]; thus enabling these bacteria to acquire ESBL by different resistance mechanisms. In recent years, the emergence of β-lactamases increased because of bacterial infections that carried β-lactamases genes [6]. Moreover, the excessive and unregulated use of antibiotics is the fundamental cause in the selection of resistance mechanisms [4]. The *Enterobacteriaceae* producers of ESBLs have become a serious problem of public health worldwide since 1995, because of the increased emergence of new variants; especially bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ genes [7,2]. In fact, the majority of the enterobacterial infections caused by ESBL producing organisms are *E. coli* and *K. pneumoniae* strains carrying the CTX-M gene responsible for community infections, especially the UTIs [8,2]. The SHV family of b-lactamases appears to be derived from *Klebsiella* spp. TEM-1, first reported from an *E. coli* isolate in 1965, has substrate and inhibition profiles similar to

those of SHV-1. *CTX* a new family of b-lactamases that preferentially hydrolyzes cefotaxime has arisen. It has been found in isolates of *Salmonella enterica* serovar, Typhimurium, *E. coli* mainly and some other species of Enterobacteriaceae. These are not very closely related to TEM or SHV b-lactamases [8]. Previously, 16S rDNA gene sequencing has been used successfully to classify and differentiate among strains of *E. coli* [9]. In the present study, we intended to conduct antibiotic susceptibility screening of *E. coli* from patients with UTIs in Abdul-Aziz hospital in Taif region, Saudi Arabia and detect the common ESBL genes. Meanwhile, no available data have been known regarding the prevalence and genotype of genes responsible for beta-lactam antibiotic resistance in *E coli* isolated from UTIs patient in Taif, Saudi Arabia. the present study was initiated to determine the prevalence and genotype the ESBL positive bacteria with 16S rDNA gene sequencing. The phenotype and genotype of ESBLs were characterized to provide useful information about the prevalence and evolution of ESBLs in *E coli*.

2. MATERIALS AND METHODS

2.1 Bacterial Culture and Identification

Our study protocol was approved by Taif University Medical Ethics Review Board (project No. 1-437-5371) in accordance with the guidelines for the protection of human subjects. Samples were collected for clinical purposes from inpatients at King Abdul-Aziz Hospital, Al-Taif, Saudi Arabia between September, 2016 to November, 2017 after patient consent documented on standard hospital forms. The clinical samples were from various origins. We received about 100 bacterial isolates anonymized by coding linked to patient identities from the clinical laboratory, 21 of them were *E. coli*, which were derived from the samples originally collected for clinical purposes. The isolates were subcultured on selective media including EMB medium specific for *E. coli* growth to differentiate between *E. coli* and other coliforms as *salmonellae* and *shigellae*, and Nutrant Agar media for storage the isolates.

2.2 Antimicrobial Susceptibility Tests

Cultured bacteria were resuspended in 0.45% saline and matched to the required McFarland units. Two milliliters of bacterial suspension were automatically loaded into a VITEK 2 microbial identification system (bioMérieux, Durham, NC, USA) for identification with gram-negative bacilli and antimicrobial susceptibility testing-GN04 cards [2]. Reference strains including *E. coli* American Type Culture Collection (ATCC) 25922 were used as controls. Criteria used to characterize ESBL are those of the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards) [10]. The gram-negative bacilli were tested for their susceptibility to Amp (Ampicillin), Am/Clav (Amox/clavulinc), Pip/Taz (piperacillin/tazobactam), Cefo (cefoxitin), Cefta (ceftazidime), Ceftr (ceftriaxone), Cefe (cefepime), Imi (imipenem), Mer (meropenem), and Cefu (cefuroxime).

2.3 Molecular Characterization of Beta-Lactamases in *E coli*.

2.3.1 DNA extraction

DNA was extracted from *E. coli* isolates using a DNeasy bacterial Mini Kit (QIAGEN) according to the manufacturer's instructions.

2.3.2 Polymerase chain reaction for detection of the ESBL-genes

The PCR was performed with the Go Taq® Green Master Mix, Promega, USA, according to the manufacturer's instructions. Three genes in ESBL-producing *E. coli* were included: bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ genes. PCR primers and conditions for each gene are described in Table 1 [11]. Expected sizes of the amplicons were ascertained by electrophoresis in 1.5 % agarose gel with an appropriate molecular size marker (100-bp DNA ladder, MBI, Fermentas, Lithuania, USA).

2.3.3 PCR amplification of 16S-rDNA gene

Amplified 16S-rDNA primers described by Hassan and Belal (2016) [12] were used for PCR amplification for identification of *E. coli* isolates. DNA (2 µl; approximately 50 ng) was used as template for the polymerase chain reaction assays. The amplifications were carried out in thermo cycler as following. 2X PCR Master Mix from (Promega®, Lithuania, USA) was purchased and used for PCR reaction. Each reaction contains all necessary reagents (dNTPs 200 nm of each, 0.6 unit of Taq DNA polymerase and 10 pmol of each primer) to amplifiy the 16S rDNA gene.

Table 1. Primer sequences and amplicon sizes of Beta-lactam genes

Primers name	Primer sequence (5′→3′)	Product size (bp)
$bla_{CTX\text{-}M\text{-}f}$	GAC GAT GTC ACT GGC TGA GC	860
$bla_{CTX\text{-}M\text{-}r}$	AGC CGC CGA CGC TAA TAC A	
$bla_{TEM\text{-}f}$	ATCAGCAATAAACCAGC	516
$bla_{TEM\text{-}r}$	CCCCGAAGAACGTTTTC	
$bla_{SHV\text{-}f}$	AGGATTGACTGCCTTTTTG	392
$bla_{SHV\text{-}r}$	ATTTGCTGATTTCGCTCG	

2.3.4 Sequencing of 16S-rRNA gene

About 1260 bp 16S rDNA fragments were purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions and sequenced with the same primers using the sequencer (Gene analyzer 3121). The bacterial 16S-rDNA sequences obtained were then aligned with known 16S-rDNA sequences in Genbank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI), and percent homology scores were generated to identify bacteria isolate. The deduced sequence was aligned using Molecular Evolutionary Genetics Analysis (MEGA) version 7.10 used for phylogenetic tree construction [13].

3. RESULTS AND DISCUSSION

3.1 Microbial Identification of *E. coli* Isolates

Typical colonies of *E. coli* on EMB agar are usually described as green-metallic sheen colonies and other gram-negative bacterium (*salmonellae* and *shigellae*) demonstrating growth with pinkish mucoid colonies. Green-metallic sheen colonies were identified as *E . coli* and selected for further studies (Fig. 1). These results confirmed with 16S rDNA gene sequencing method.

3.2 Antimicrobial Susceptibility Tests

Twenty one *E. coli* isolated from inpatients at King Abdul-Aziz Hospital, Al-Taif, Saudi Arabia were tested for their antimicrobial susceptibility against 10 antibiotics (Fig. 2 and Table 2). The overall susceptibility, intermediate and resistance were determined. A large percentage of the *E. coli* was susceptible to Imipenem (100%), Ampicillin (90%) and cefoxitin, but resistant to cefepime (38%). PCR amplification of *bla*-genes was performed. Intermediate resistances were observed for most antibiotics and it was relatively higher for amoxycillin/clavulanic acid (19%). These results are agreement with [14,15,2,16, 17]. It is also indicated that in clinical diagnoses patients with sensitive antibiotic can be given a good results. Antibiotics play very important role in decreasing diseases, illness and/or death associated with bacterial infections patients with UTIs.

3.3 Detection of Beta-Lactamases Genes in *E. coli*

In the present study, the majority of the $bla_{CTX\text{-}M}$, bla_{TEM}, type ESBLs were predominant (21/21) followed by bla_{SHV} (18/21). *E. coli* isolates were positive for $bla_{CTX\text{-}M}$ genes from CTX-M group 1. Only 18 *E. coli* isolate was positive for the bla_{SHV} gene (Fig. 1 and Table 3). *E. coli* isolate 2 (negative for bla_{SHV} positive for bla_{TEM}) was more resistant than the other *E. coli* isolates because

Table 2. Antibiotic sensitivity pattern among *E. coli* isolates.

	Antibiotic sensitivity percentage								
	Amp	Am/Cla	pip/taz	Cefo	Cefta	Cefe	Imi	Cip	Cefu
R	10	14	5	15	29	38	0.0	46	33
S	90	67	76	85	62	62	100	54	67
I	0.0	19	19	0.0	9.0	0.0	0.0	0.0	0.0

Amp (Ampicillin), Am/Clav (Amox/clavulinc), Pip/Taz (piperacillin/tazobactam), Cefo (cefoxitin), Cefta (ceftazidime), Ceftr (ceftriaxone), Cefe (cefepime),Imi (imipenem), Mer (meropenem), Cip (ciproflox), Cefu (cefuroxime), S (Sensitive), R (Resistant), I (Intermediate).

they showed resistance to cefepime (38%) (Table 3 and Fig. 3). In agreement with our results Anago et al. [18], found that the phenotypic screening for ESBL was realized by resistance to cephalosporin with the presence of genotype TEM and SHV could not predict the resistance pattern to them. The difference in the beta-lactame gene was reported from different auhors, in a Iran study, the TEM gene predominated followed by SHV [19,20]. A report from Canada showed SHV as the main group of ESBLs. However, reports from South America, Palastien, Spain, New York, the United Kingdom, and several parts of Saudi Arabia subcontinent revealed CTX-M as the predominant gene [2, 21]. Until the year 2000, TEM was the most prevalent ESBL gene in the Indian bacterial population but was replaced by CTX-M in the following decade [22]. In urine isolates in our setting, TEM was again predominant. The present study clearly demonstrates the dramatic change in the gene pool in Indian *Enterobacteriaceae*. In our study, 22 (55%) of phenotypically positive ESBL strains lacked TEM, SHV, and/or CTX-M genes, which can be explained by the possible presence of other ESBL-encoding genes in the studied Indian bacterial population. [1,19,23]. The differences between our study results and those of other authors indicated that the prevalence and type of ESBL genes may vary from one geographical region to another. Because of the increased complexity of β-lactam resistance in gram-negative organisms, the key to effective surveillance is the use of both phenotypic and genotypic analyses in concert [24]. The expression of beta-lactamase genes depends upon the environmental conditions such as the presence of antibiotics, and gene presence detected by PCR does not necessarily indicate its expression. Studies have revealed that some of these “hidden ESBL” genes can be detected through modifications in the phenotypic ESBL confirmatory tests [19,20].

3.4 Sequencing of 16S-rDNA Gene

Ribosomal operons are great relevance for the study of bacterial evolution and phylogeny [23], therefore, sequencing of 16S rDNA has been widely used to re-construct phylogenetic relationships of microorganisms [5]. Phylogenetic analysis of the partial 16S rDNA sequences from *E. coli* strains were shown in (Fig. 4). The genetic proximity between *E. coli* strains was also observed by [25,21,22,2] using sequencing of approximately 1200 bp of the 16S rDNA, thereby highlighting the low level of variability of these organisms.

Table 3. PCR results of genes encoding important β-lactamases (bla) genes in *E. coli* isolates

Bacterial isolates	*bla-genes*		
	CTX-M	TEM	SHV
E. coli-1	+	+	+
E. coli-2	+	+	–
E. coli-3	+	+	+
E. coli-4	+	+	+
E. coli-5	+	+	+
E. coli-6	+	+	+
E. coli-7	+	+	+
E. coli-8	+	+	–
E. coli-9	+	+	+
E. coli-10	+	+	+
E. coli-11	+	+	+
E. coli-12	+	+	+
E. coli-13	+	+	–
E. coli-14	+	+	+
E. coli-15	+	+	+
E. coli-16	+	+	+
E. coli-17	+	+	+
E. coli-18	+	+	+
E. coli-19	+	+	+
E. coli-20	+	+	+
E. coli-21	+	+	+

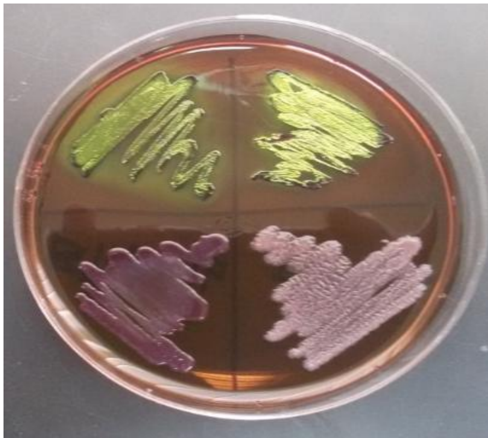

Fig. 1. EMB agar inoculated with *E. coli* (a gram-negative bacterium) demonstrating growth with green-metallic sheen colonies and other gram-negative bacterium (not *E. coli*) demonstrating growth with pinkish mucoid colonies

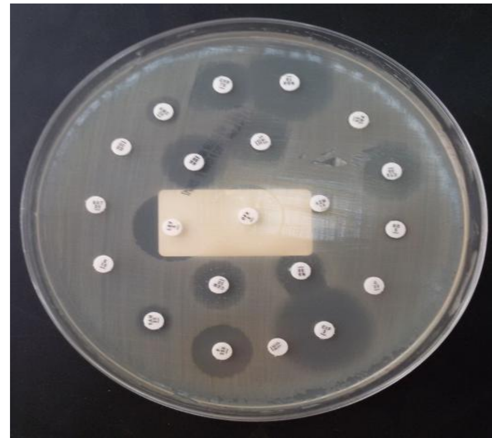

Fig. 2. The combination disk diffusion test of antibiotic resistance in the *E. coli* strain

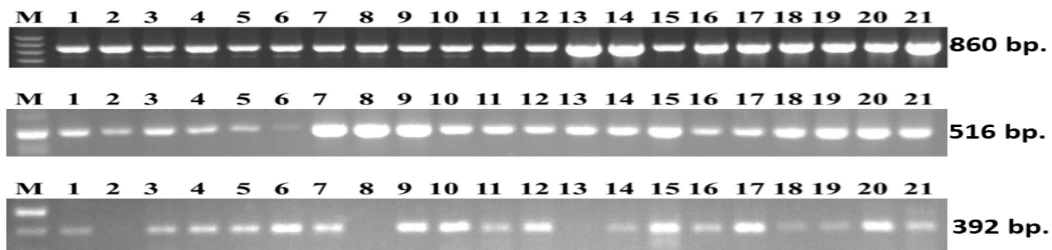

Fig. 3. Amplification of β-lactamases (bla) genes in *E. coli* isolates by single PCR. (a) Amplification of $bla_{CTX\text{-}M}$ gene (860 bp). (b) Amplification of bla_{TEM} gene (520 bp). (c) Amplification of bla_{SHV} gene (400 bp). M: 100-bp DNA ladder.

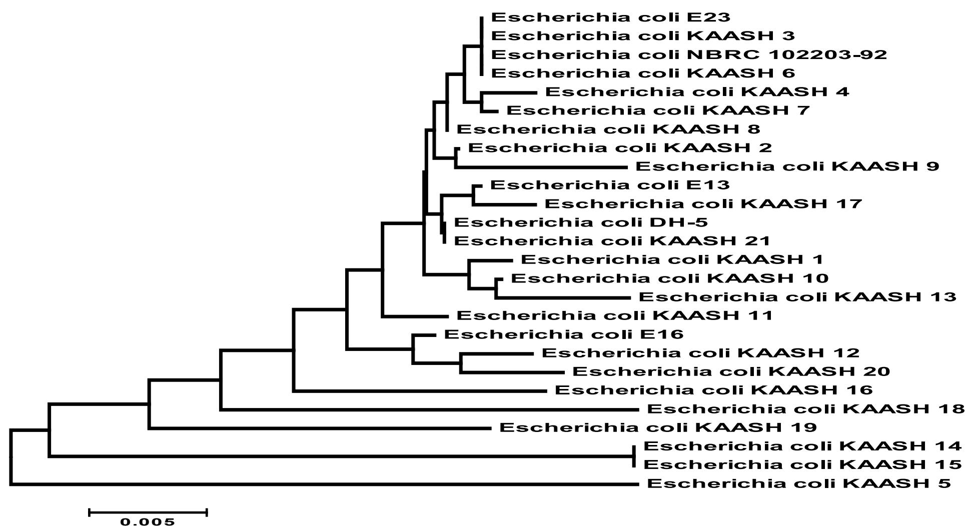

Fig. 4. Neighbor-joining phylogeny based on 16S rRNA gene sequences of 21 *E. coli* isolates and related *E. coli* strains obtained from a BLAST search of the NCBI database.

4. CONCLUSION

From the present data, we can conclude that UTI *E. coli* isolates was susceptible to Imipenem (100%), Ampicillin (90%) and Cefoxitin (85%), but resistant to Cefepime (38%), which were closely associated with existence of selected *bla*-genes (bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$) harbored by diverse plasmids among a highly diverse population of *E. coli* strains. thus, these data suggest that antibiotics resistance genes has evolved for some time in Saudi Arabia and that a huge reservoir for resistance is maintained in the community.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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