

# Are TEM and CTX Beta-Lactamase Genes Common among Urinary Isolates from Ain Shams Hospital Intensive Care units?

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## ABSTRACT

The extended-spectrum  $\beta$ -lactamase producing Gram Negative bacteria are widely spread worldwide. Productions of these enzymes cause bacterial resistance to beta-lactam antibiotics as penicillin and cephalosporins. These enzymes act by hydrolyzing these antibiotics. Many ESBL variants are known that have been classified into nine different structural and evolutionary families based upon alteration of the amino acid configuration around the enzyme active site such as TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA, and OXA.

**Aim:** To determine the distribution of ESBL (blaTEM and blaCTX-M) genes among the Gram negative isolates collected from urine samples in Intensive care units of Ain Shams University Hospitals.

**Materials and Methods:** The study was conducted on 40 Gram negative isolates, isolated from urine samples from Intensive care units of Ain Shams University Hospitals from July 2018 till December 2018. All the isolates were subjected to conventional microbiological identification methods, Antimicrobial susceptibility testing by disc diffusion test, followed by Polymerase chain reaction for detection of blaTEM and blaCTX-M ESBL genes.

**Results:** In this study, Phenotypic detection of ESBL among the isolates showed that 8 (20%) isolates were ESBL producers by double disc method and 32 (80%) non ESBL producers. ESBL gene (blaTEM) was found in 15 isolates (37.5%) and ESBL gene (blaCTX-M) was found in 8 isolates (20%) and five isolates were positive for both genes.

**Conclusion:** Traditional Phenotypic tests for ESBL production cannot detect the ESBL subtype and cannot detect those genes whose expression is masked. Therefore, the genotypic method is suggested as the method of choice for detection of ESBL-producing strains among gram negative bacteria. The distribution of blaTEM was (37.5%) and blaCTX-M was (20%) and five isolates were positive for both genes.

**Keywords:** Antimicrobial susceptibility testing; Antibiotics; bla CTX-M; ESBL

## ABBREVIATIONS

ESBLs: Extended Spectrum  $\beta$ -Lactamases; PCR: Polymerase Chain Reaction; GNB: Gram-Negative Bacilli

## INTRODUCTION

Multidrug resistance is now increasing worldwide among bacteria specially gram negative ones, causing both community-acquired and hospital acquired infections [1]. Multidrug resistance organisms can cause life threatening infections worldwide. The enterobacteriaceae family and other gram negative bacilli are mainly responsible for many cases of these antibiotic resistance, mainly by the means of Extended Spectrum  $\beta$ -Lactamases (ESBLs) production [2]. ESBLs are derived from genes (TEM-1, TEM-2, or SHV-1) by mutations that alter the amino acid configuration around the enzyme active site. ESBLs were first discovered in 1983

[3]. 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 [4]. ESBLs are typically plasmid-mediated enzymes that hydrolyze the penicillins, the third generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime) and the monobactam (aztreonam). They are not active against the cephamycins (cephoxitin and cefotetan) and carbapenems (imipenem, meropenem) but are susceptible to  $\beta$ -lactamase inhibitors such as clavulanic acid and tazobactam [5]. Gram negative bacilli is now considered as one of the major

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causes of urinary tract infections (UTIs) [6]. Also ESBL-producing *E. coli* is considered as one of the most multidrug resistant strains emerging worldwide [7]. A variety of ESBLs, mostly of the genotypes CTX-M, TEM and SHV types, had been reported in members of enterobacteriaceae family, and are often undetectable by the current isolation and susceptibility methods [8]. For detection of ESBL production, determination of susceptibility to cephalosporin followed by inhibition of the ESBL activity, by clavulanic acid or tazobactam is usually done [9]. Or using the double-disk synergy test with 80 to 90% sensitivities and specificities [10]. Molecular detection of ESBL genes by PCR, hybridization, and sequencing is an alternative accurate method [11]. Although conventional microbiological methods for detection of antimicrobial resistance are reliable, yet they need several days to be completed so rapid detection of resistant genes using molecular methods as PCR might help in rapid patient treatment [12,13].

## MATERIALS AND METHODS

The study was conducted on 40 gram negative isolates, isolated from urine samples from intensive care units of Ain Shams University Hospitals from July 2018 till December 2018. All the isolates were subjected to conventional microbiological identification methods, Antimicrobial susceptibility testing by disc diffusion test, results were interpreted according to Clinical and Laboratory Standards Institute 2018, followed by PCR for detection of blaTEM and blaCTX-M ESBL genes.

### Bacterial isolation and identification

A total of 40 non-duplicate Gram-Negative Bacilli isolates were included in the current study. The isolates were identified based on the colony morphology on nutrient agar, blood agar and MacConkey agar and were confirmed using biochemical tests.

### Antimicrobial susceptibility testing

Antimicrobial susceptibility testing by disc diffusion test were done for all isolates using different antibiotics (Oxoid, England): Ampicillin (AM, 10 µg) Amoxicillin/Clavulanic acid (AMC, 20/10 µg), Piperacillin/Tazobactam (TPZ,100/10 µg), Aztreonam (ATM, 30 µg), Cefazidime (CAZ, 30 µg), Cefotaxime (CTX, 30 µg) Cefoxitin (FOX, 30 µg), Cefepime (FEP, 30 µg), Meropenem (MEM,10 µg), Gentamicin (CN,10 µg), Amikacin (AK, 30 µg) Ciprofloxacin (CIP,5 µg), Trimethoprim-sulfamethoxazole (SXT, 25µg). The interpretation of results were done according to CLSI, 2018 [14].

### Phenotypic detection of ESBL

All isolates were phenotypically tested for ESBL production by double disk synergy test: The procedures were done in a way like disc diffusion method. Commercially prepared antibiotic discs 6 mm in diameter of Cefazidime (CAZ) 30 µg were placed with Cefazidime/Clavulanic acid (AMC) 30/10 µg using a sterile forceps on plates of Mueller-Hinton agar that were previously inoculated by the desired bacteria, then incubated at 37°C for 24 hours. An organism was considered as ESBL producer if there was a ≥ 5 mm increase in the zone diameter of Cefazidime/Clavulanic acid disc and that of ceftazidime disc alone. The phenotypically confirmed ESBL and non-ESBL isolates were tested genotypically by performing Polymerase Chain Reaction (PCR), using primers specific for the detection of blaTEM, and blaCTX-M genes.

### Molecular detection of bla TEM, and bla CTX-M genes

Total extraction of DNA: Total plasmid DNA was prepared [15]. Bacterial isolate was grown in 15 ml of liquid medium (nutrient broth media) at 37°C with continuous shaking at 250 rpm. Cell pellet were collected by centrifugation at 13000 rpm for 4 min. and washed by distilled water. Pellets were frozen at 4°C overnight and then let in room temperature until thawing. Pellets were suspended in 200 µl of solution "A" (10 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 50 mM NaCl, and 20% (w/v) sucrose) containing 2 mg/ml freshly prepared lysozyme and incubated for 45min at 37°C to lyses the cells. Added 400 µl of solution "B" (0.2 M NaOH and 1% (w/v) SDS) was added to cell lysate, shaken gently and kept on ice for 5 min. Next added 300 µl of solution "C" (potassium acetate pH 5.5, Glacial acetic acid) mixed well and kept on ice for 5 minutes, centrifugation at 13000 rpm for 5 min to remove the remained of cell lysate. Add equal volume of phenol-chloroform- isoamyl solution. The solution mixed by inverting several times and left at room temperature for few minutes for the phase separations. Centrifugation at 10000 rpm for 5 minutes until the cellular protein formed an interphase layer between organic and aqueous phase, upper aqueous phase containing DNA. Transfer upper aqueous phase in clean eppendorf and re-extracted with phenol solution two to three times until no protein interphase layer. Collect the clear supernatant containing DNA in clean sterilized Eppendorf and added 0.6 volume of isopropanol to precipitate DNA left for about 2 hours. DNA pellet was collected by centrifugation at 13000 rpm for 15 min. DNA pellets were washed with 70% cold ethanol then washed by sterilized dist. water (sdH<sub>2</sub>O) and dried. According to size of DNA pellet dissolve in the volume of sdH<sub>2</sub>O and left for an hour before being analyzed by gel agarose electrophoresis.

### Polymerase Chain Reaction (PCR)

This in vitro method for enzymatic amplification of specific DNA sequences uses two oligonucleotides that hybridize to opposite strands and flank the region of interest in the target DNA. The PCR was performed in 25 µl reaction volume containing: 1X buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl<sub>2</sub>), 250µM each of dGTP, dATP, dCTP and dTTP, 0.5 units of Taq DNA polymerase, 100 pmol of each primer and the DNA template. Purified DNA or bacterial colonies were used in PCR to detect the presence or absence of blaTEM, and blaCTX-M genes. When bacterial colonies were used, they been boiled in water bath for five minutes followed by cooling for five minutes, then centrifugation at 15000 rpm for two minutes and the supernatants were used directly as DNA template to perform PCR. Components of the PCR were mixed thoroughly and overlaid with a drop of mineral oil to prevent evaporation, DNA amplification was carried out in the thermal cycles with an initial DNA denaturation step for 5 min at 94°C followed by 35 cycles. Each cycle consisted of: Denaturation for 1 min at 94°C, annealing for 45 sec at 50°C and extension at 72°C for 3 min and a final extension step for 7 min at 72°C at the end of the 35 cycles before analyzing the PCR products in agarose gel by electrophoresis. The product size was 931 bp for bla TEM and 909 bp for blaCTX-M genes

### Statistical analysis

Statistical analysis was done on a personal computer using the Statistical Package for Social Sciences (SPSS) version 17 as follow:

- Descriptive statistics:
- Frequency number and percentage for qualitative data.
- Analytical statistics:

-Paired t-test used to compare between related samples (pre and post).

-Level of significance considered at 0.05 i.e.:

P value > 0.05 non-significant.

P value ≤ 0.05 significant.

P value ≤ 0.01 highly significant

## RESULTS

A forty-gram negative clinical isolates were obtained from urine samples from patients (21 female and 19 male) in Intensive care units of Ain Shams University Hospitals from July 2018 till December 2018

The 40 clinical isolates were identified by conventional microbiological methods, they were 10 (25.0%) Ecoli, 21 (52.5%) Klebseilla spp, 6 (15.0%) pseudomonas spp, 3 (7.5%) Citrobacter (Figures 1-3).

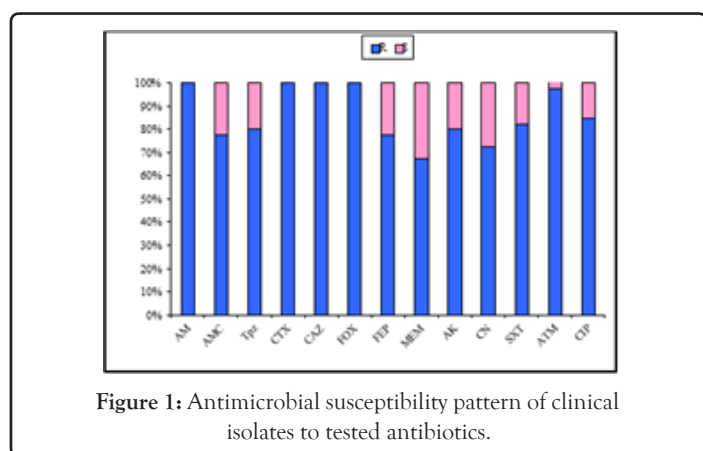


Figure 1: Antimicrobial susceptibility pattern of clinical isolates to tested antibiotics.

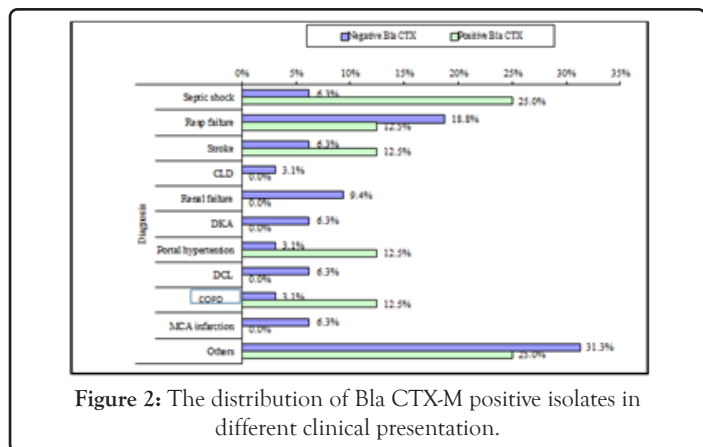


Figure 2: The distribution of Bla CTX-M positive isolates in different clinical presentation.

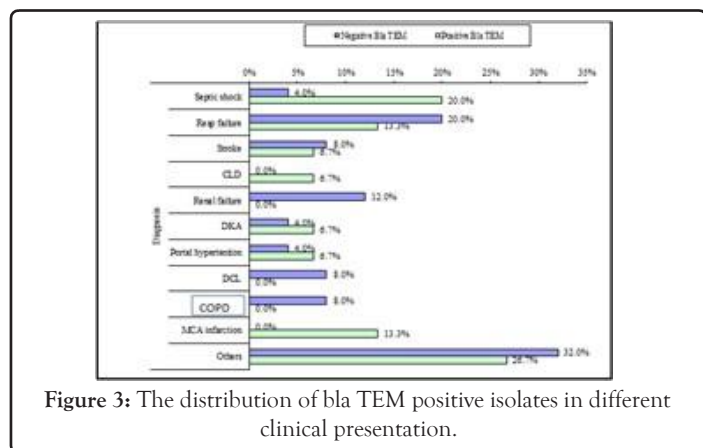


Figure 3: The distribution of bla TEM positive isolates in different clinical presentation.

Then antimicrobial susceptibility testing by disc diffusion test were done for all isolates using different antibiotics , Ampicillin (AM,10 µg) Amoxicillin/Clavulanic acid (AMC, 20/10 µg) piperacillin/tazobactam (TPZ,100/10 µg), Aztreonam (ATM, 30 µg), Ceftazidime (CAZ, 30 µg), cefotaxime (CTX,30 µg), Cefoxitin (FOX,30 µg),cefepime (FEP,30 µg), meropenem (MEM,10 µg), gentamicin (CN,10 µg), amikacin (AK,30 µg), ciprofloxacin (CIP,5 µg), Trimethoprim-sulfamethoxazole (SXT, 25 µg) , The interpretation of results were done according to CLSI, 2018.

ESBL detection were done using double disc synergy using Ceftazidime (CAZ) 30 µg and Ceftazidime/Clavulanic acid (AMC) 30/10 µg, There were 8 isolates (20%) positive for ESBL production by using this method and 32 isolates (80%) were negative for ESBLproduction.

All isolates were tested for the presence of bla TEM and bla CTX-M genes by using PCR. Bla TEM gene was present in 15 isolates (37.5%) and bla CTX-M gene was present in 8 isolates (20%) and five isolates were positive for both genes

Bla CTX-M gene were present in 8 isolates (20%), four isolates (50.0%) were E. coli, 3 isolates (37.5 %) were Klebseilla spp and 1 isolate (12.5%) was pseudomonas. Among the 8 isolates 3 only were confirmed phenotypically as ESBL producers, with no statistical significance was detected

There is no statistical significance association as regards the clinical presentation of patients and the presence of Bla CTX-M genes in the clinical isolates.

Regarding antibiotic use there is statistical significance between use of vancomycin and amoxicillin clavulanate and the presence of Bla CTX-M in clinical isolates.

Bla TEM gene was present in 15 isolates (37.5%), three isolates (20.0%) were E coli, 9 isolates (60 %) were Klebseilla spp and 3 isolates (20%) were pseudomonas. Among the 15 isolates 5 only were confirmed phenotypically as ESBL producers, but there is no statistical significance difference detected.

There is no statistical significance association between clinical presentation of patients and presence of Bla TEM gene in clinical isolates,

Regarding antibiotic use there is no statistical significance between use of antibiotics and the presence of bla TEM in clinical isolates.

## DISCUSSION

In our study, phenotypic detection of ESBL among the isolates showed that 8 (20%) isolates were ESBL producers by double disc method and 32(80%) non ESBL producers. ESBL gene that predominated among isolates was blaTEM (15 isolates (37.5%) followed by blaCTX-M (8 isolates (20%), and five isolates were positive for both genes [16,17]. The predominant gene was TEM (87.1% TEM, followed by 70.6% SHV) also in accordance to another study that stated that TEM (48.7%) gene predominated the SHV (7.6%) and CTX-M (5.1%) genes responsible for ESBL production.

Studies performed all over the world showed variable results. A study was done in China showed that the TEM gene predominated followed by SHV. While studies done in Canada showed the predominance of SHV. On the other hand, studies done in South America, Spain, the United Kingdom, and India revealed CTX-M

as the predominant gene. 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.

Further, in the present study ESBL genes were detected among 8 phenotypically confirmed ESBL producers and 15 phenotypically confirmed non-ESBL producers with the predominance of bla<sub>TEM</sub> gene, and five isolated carried both genes. Similar results were detected detected 20 (52.6%) (genotypically positive) out of 38 phenotypically non-ESBL isolates carried TEM gene. Of these, 18 (90%) isolates carried TEM gene alone while 2 (10%) of them carried TEM in association with CTX-M. The difference detected between the two different methods (phenotypic and genotypic) might reflect the lower sensitivity of phenotypic method and the influence of environmental factors on the incidence of resistance. in the detection of ESBL-positive isolates.

Some ESBLs might not reach the level of detection by disk diffusion tests, leading to failure in the patient treatment. In our study it was evident that there was lack of correlation between ESBL production and disk diffusion susceptibility results that enforced the need for an improved method of ESBL detection to be incorporated in routine susceptibility procedures. In contrast, genotypic method using specific PCR amplification of resistance genes seems to have higher specificity and sensitivity. Our study showed the presence of bla<sub>TEM</sub> in 15 isolates (37.5%) followed by bla<sub>CTX-M</sub> (8 isolates (20%)), but the another study done showed that bla<sub>TEM</sub> in all 38 genotypically positive isolates and none of the isolates was detected to carry SHV or CTX-M gene alone. In addition, none of the isolates had SHV and CTX-M together or TEM, SHV, and CTX-M genes in combination.

Regarding the antibiotic susceptibility testing among genotypically confirmed ESBL producers, in this study the isolates were highly resistant to ampicillin, piperacillin/tazobactam and less resistant to and trimethoprim-sulfamethoxazole, gentamicin, amikacin and meropenem that reported that The ESBL producers were highly susceptible to imipenem, nitrofurantoin but less susceptible to ampicillin and ticarcillin. Most of the isolated Gram-Negative Bacilli (GNB) were sensitive to imipenem and amikacin.

## CONCLUSION

Incorrect detection of antibiotic resistance can lead to wrong antibiotic prescription, leading for new resistance genes. Phenotypic tests for ESBL detection only confirm whether an ESBL is produced but cannot detect the ESBL subtype and cannot detect those genes whose expression is hidden or masked. Therefore, the genotypic method is suggested as the method of choice for detection of ESBL-producing strains among gram negative bacteria. Molecular methods are sensitive, but they are expensive and require specialized equipment and expertise. The presence of TEM gene in the genotypically positive isolates indicates that this gene can be an appropriate candidate for molecular screening of ESBL-positive samples in our settings.

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