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Phenotypic and Genotypic Detection of ESBL Isolates from UTI Suffering Patients

Manar .A.kamal

Qanat.M.Atiya

College of science, Tikrit University,Tikrit,Iraq

Assist.Prof.Dr.Collegeofscience,Tikrit University,Tikrit,Iraq

Summary

Out of 115 *E.coli* isolates from UTI patient submitted to phenotypic and genotypic detection through the using of screening method and confirmation method (phenotypic method),out of 50 suspectes ESBL isolates from screening test confirm by confirmation test which revealed only 29 isolates was ESBL,positive ESBL isolated detected by phenotypic method confirm by genotypic method through detect the presence of three plasmid type bla(CTX,TEM,SHV).only bla-CTX gene was present in 29 isolates with completely absence of TEM and SHV gene.

Introduction

The term of Extended-spectrum lactamase mean (ESBLs) are enzymes with a wide range of functions. Third-generation cephalosporin resistance can be conferred by two forms of beta-lactamases Clavulanic acid does not block beta-lactamases mediated by the chromosome.which are inducible. These enzymes confer non-transferable resistance.The second kind of enzyme is plasmid-mediated beta-lactamases, which are blocked by clavulanic acid. (Weldhagen.,2004).more than 200 different clavulanic acid-inhibited enzyme variations have been discovered all over the world. Changes in the primary amino acid sequence of the enzyme are caused by point mutations in the blaTEM, blaSHV, or blaCTX genes, which are the most prevalent extended-spectrum symptoms (Bush.,2001).Because Many organisms with ESBLs are also resistant to other antibiotic classes, and these genes are usually found on plasmids., including aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and sulfonamides (Nathisuwan*etal*.,2001).

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Material and method

(1) ESBL screening test:-

According to CLSI standards, the disk-diffusion method for ESBL screening can be used with ceftazidime, aztreonam, cefotaxime, and ceftriaxone (table 1). Because ESBLs' affinity for different substrates varies, using more than one of these agents for screening improves detection sensitivity. The combination of cefotaxime (or ceftriaxone) with ceftazidime, on the other hand, is sufficient.as show in table (1)

Table (1) antibiotics used in screening test

CLSI recommended		
Antibiotic disk		**Conduct ESBL-testing if**
Cefotaxime	**30mg**	**Inhibition zone ≤ 27 mm**
Ceftriaxone	**30mg**	**Inhibition zone ≤ 25 mm**
ceftazidme	**30mg**	**Inhibition zone ≤ 22 mm**
aztreonam	**30mg**	**Inhibition zone ≤ 27 mm**

(2)confirmation test:-

Enterobacteriaceae that are suspected of producing ESBL enzymes can be tested using the Combination Disc Test (CDT) and/or Double-Disc Synergy Test (DDST). These assays can be used to see if Clavulanic acid inhibits ESBL activity.

(3)Double-Disc Synergy Test (DDST):-

Synergy Test with Two Discs (DDST) Cephalosporin (cefotaxime or ceftriaxone, ceftazidime, cefepime)-containing discs are placed adjacent to clavulanic acid, amoxicillin + clavulanic acid, or ticarcillin + clavulanic acid-containing discs. The inhibition zones around any of the cephalosporin discs are enhanced in the direction of the disc containing clavulanic acid, indicating a positive result. The distance between the discs is crucial, and for cephalosporin 30 g discs, a center-to-center gap of 20 mm has been determined to be ideal; however, it can be reduced (15 mm) or widened (30 mm) for strains with very high or low

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resistance levels, respectively.

Table (2) antibiotics used in DDST

Double disk synergy test	**Cefotaxime,Ceftriaxone,Ceftazidime,Cefepme (30 µg)**	**expansion of indicator cephalosporin inhibition zone towards antibiotic with Clavulanic acid**
	Amoxicillin + Clavulanic acid(AUG)20+10 µg	
	Ticarcillin + Clavulanic acid(TTC) 75+10 µg	

(3) Genetic assay

Dna plasmid isolation

Dna plasmid extracted according to the kit procedure (Bio diamond).

1- Transfer 1.5 ml of broth containing cells into 1.5 ml centrifuge tubes and centrifuge at 13000 rpm for 1 minute to harvest and lyse bacteria. Supernatant should be discarded.

2 -Re-suspension the pellet cells in 200 l of s1/RNaseresuspension solution. To mix, pipette up and down or vortex.

3- Pour 200 l of Lysis Solution SII into the mixture. To combine, gently invert. Do not create a vortex. Allow for a 5-minute clearing period.

4-Make the clear lysate by adding 300 liters of neutralization solution (SIII). To combine, invert 4-6 times.

5-Centrifuge for 3 minutes at 14,000 rpm.

6-Prepare the binding column by placing the sp Column in a collection tube (apply the supernatant from step4 to the column).(

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7- Spin for 30 seconds @ 14,000 rpm. Flow the trash through

8- Wash the column with 400 l Wash solution to remove contaminants (W1). Spin @ 13000rpm for 30 seconds. Allow the waste to flow freely.

9- add 600µl of buffer II(WII) ethanol added in to the sp column back into the same collection tube .centrifuge at 14000 rpm for 2min.

10- Transfer the column to a new collecting tube for elution of pure Plasmid DNA. 70 liters of Elution Solution (or sterile water). For 2 minutes, spin at 14,000 rpm for 1 minute. Plasmid DNA can be found in the eluate. Plasmid DNA should be stored between 20 and 80 degrees Celsius.

PCR Reactions:-

The amplification of genes for extend spectrum beta lactamase from 30 *E. coli* plasmid isolates was carried out using a polymerase chain reaction (PCR). Jena – Bioscience Company offered specialized primers. Oligonucleotides used in this study and their sequences are listed in (table 3).

Table(3)The Sequence of Primers and Size of Amplified Products

primer	Oligonucleotide sequences	Size of amplicons	Reference
Bla TEM	***Forward*- 5'- GAG ACA ATA AAC CCT GGT AAA T- 3'** ***Reverse*- 5'- AGA AGT AAG TTG GCA GCA GTG- 3'**	**508 bp**	**Siqueira*et al.*, 2009**
Bla CTX	***Forward*-5'- GAA GGT CAT CAA GAA GGT GCG- 3'** ***Reverse*- 5'-GCA TTG CCA CGC TTT TCA TAG - 3'**	**328 bp**	**Siqueira*et al.*, 2009**
Bla SHV	***Forward*-5'- GTC AGC GAA AAA CAC CTT GCC C- 3'** ***Reverse*- 5'-GTC TTA TCG GCG ATA AAC CAG - 3'**	**410 bp**	**Rahman and Deka, 2014**

To prepare pcr reaction the total volume must be adjusted to 20 µL and this done as follow :- Mixing 2 µlof mastermix with 2 µl of primer (forwed&reverse) oneµlfor each ,and 2 µl of DNA template finally the total volume adjusted to 20µl through the adding 14µl of deionized water.

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Table(4) PCR conditions for detection of UPEC virulence genes.

Initial denaturation	**95c**	**5min**	
Denaturation	**95c**	**30sec**	**35cycle**
Annealing	**55 C for TEM ,66C for SHV and CTX**	**30sec**	
Elongation	**72C**	**2min**	
Final elongation	**72C**	**10min**	

Result

out of 50 isolates suspected ESBL resist third generation of cephalosporin and aztreonam submitted to screening test and confirmation test and recommended by CLSI only 30 isolated exhibit inhibition zone less than 27 mm for cefotaxime and aztreonam and 25,22mm for ceftriaxone and ceftazidime and demonstrate that the inhibitory zones surrounding either of the cephalosporin discs are enhanced in the direction of the DDST disc containing clavulanic acid.

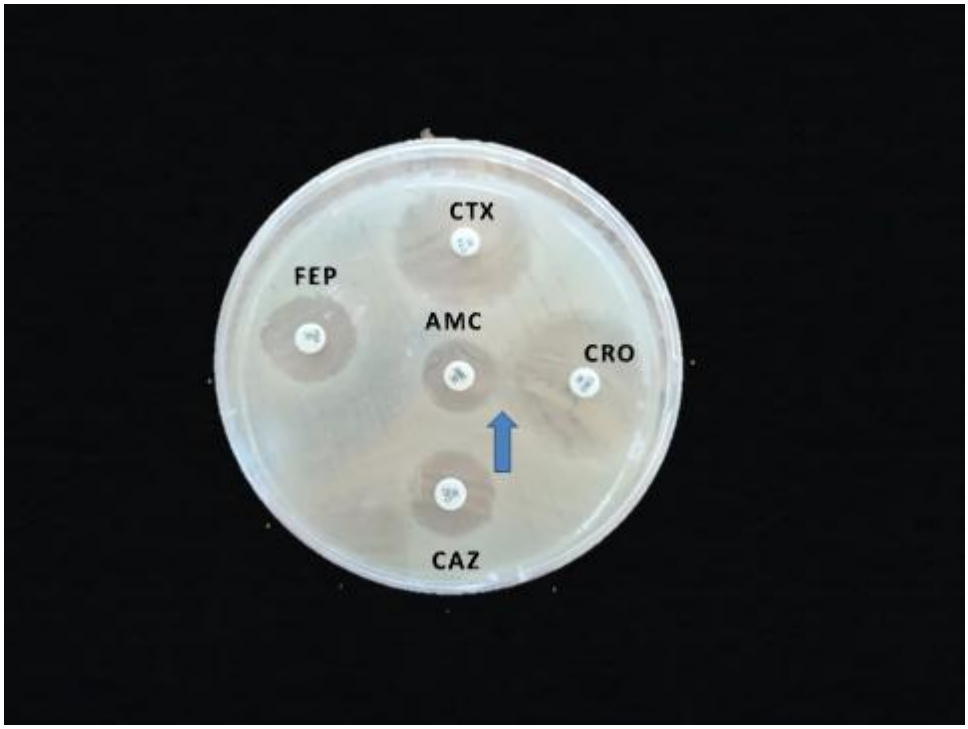

Figure (1) double disk synergy test

Three primerused in the detection of bla(CTX,SHV,TEM) among 30 phenotypic detected ESBL our result show only CTX present among 19(63.6%) isolates with absence completely

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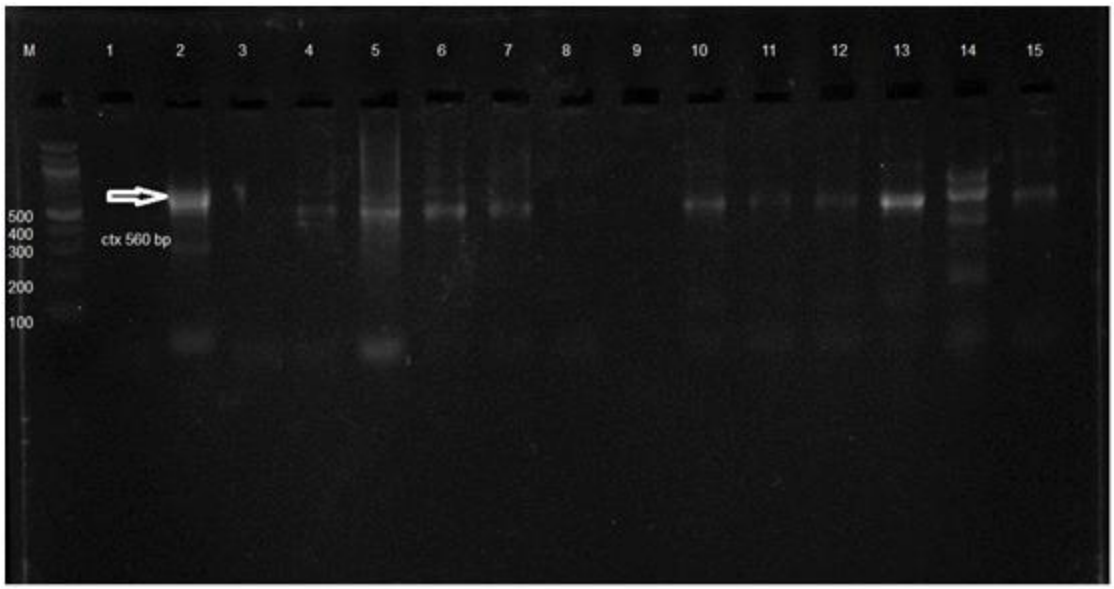
of SHV and TEM ,also 11 isolated show don’t cary any gene



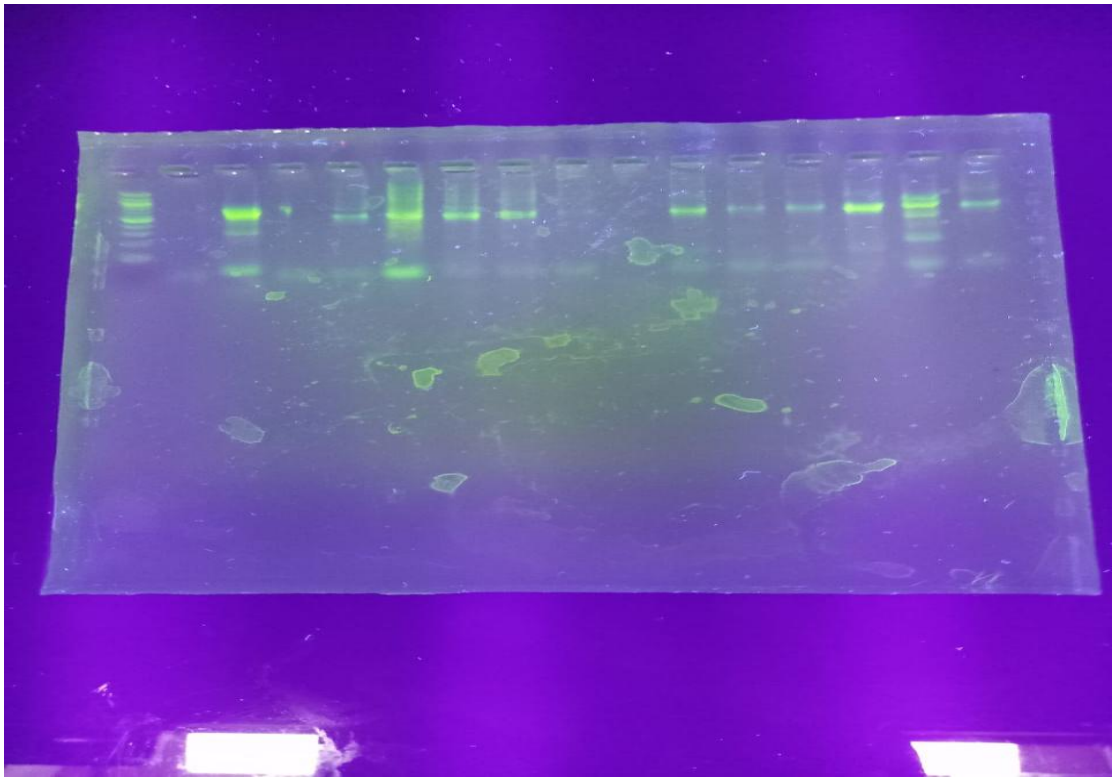


Figure (2) CTX gene demonstrated by gel decomentation .

Discussion

Despite the fact that this investigation found a modest number of ESBL generating *E. coli*uropathogen isolates, [26%(30/115], I think this low percent result from in the phenotypic test someESBL fail to reach a detectable level through disk diffusion. Several other research

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look on the phenotypic approach's sensitivity as well as environmental variation may affect the occurrence of the resistance ,furthermore ESBL perhaps express chromosomally or AmpC beta lactamase ,thus ESBL-producing strainswithAmpC beta lactamase may result in the creation of false –negative ESBLs also this problem lead to fail in the treatment of the infected patients(Collee and Miles.,1996). The frequency of ESBL generating E. coli uropathogens varied by area. Spandafino et al (2014) discovered an ESBL generating *E. coli* rate of 12.5 % in the United States, New York, whereas Subashini and Krishnan (2013) discovered a prevalence of 4.5 % in India. ESBL *E. coli* generating isolates are being found all over the world, and their incidence is increasing (Osthoff*et al.,* 2015).

Also I think the reasons for absence of genes among 11 isolates these may have ahiddengene,our result agree withand by Ahmed *et al*., 2013., in which CTX was the most prevalent gene (71.4 %) in *E. coli*, and contradicts the findings of Eftekhar*et al.,* 2012, in which SHV (43.1%) outnumbered TEM (35.2 %), Several additional research conducted throughout the world yielded mixed findings. The TEM gene predominated in a Chinese study, followed by SHV. According to a Canadian research, SHV is the most common kind of ESBL. CTX was demonstrated to be the dominant gene InsSouth America, Sspain, Nnew York, the United Kingdom, and other regions of the iIndian subcontinent, Up until 2000, the most prevalent ESBL gene in iIndian bacteria was TEM when it was surpassed by CTXin in the following decade. The differences in prevalence and kind of ESBL genes by geographical region that we found between our findings and those of other studies.

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