

Rapid detection of Extended spectrum β -lactamase (ESBL) producing strains of *Escherichia coli* in urinary tract infections patients in Benha University Hospital

ABSTRACT

The aim of this study was done to detect the prevalence of ESBL, AmpC producing and ESBL&AmpC co-producing strains of *Escherichia coli* (*E. coli*) in urinary tract infections patients in Benha University Hospital and to evaluate the performance of CHROMagar™ ESBL media for rapid screening of ESBL producing *E. coli*. All patients under study were subjected to: Full history taking and clinical examination. Bacteriological study included; urine sample collection from each patient and subjected to urine analysis, urine culture on cysteine lactose electrolyte deficient agar (CLED) agar, CHROMagar™ ESBL media and MacConkey agar supplemented with 2 mg/liter ceftazidime (MCKC). ESBL detection in *E. coli* isolated on CLED agar by phenotypic screening by clinical and laboratory standards institute (CLSI) method then phenotypic confirmation by E. test. The presence AmpC beta-lactamase ESBL was detected by AmpC disc test and detection of AmpC beta-lactamase & ESBL co-producers by cefepime & Cefepime+ Clavulanate E test. In this study out of 45 *E. coli* strains 24 (53.3%) ESBL producers were detected by E. test (golden method for confirmation of ESBL according to CLSI) and 21(46.7%) strains were non ESBL producers. Out of the 24 isolated ESBL producing *E.coli* strains 9 (37.5%) were detected in community acquired UTI patients while 15 (62.5%) were detected in health care associated UTI patients. The sensitivity of the MCKC and CHROMagar™ ESBL media were 100%. While specificity were 80.8% and 87.5% respectively. In our study out of 45 isolated *E.coli* strains 14 (31.1%) were AmpC producers by AmpC test, 4 (8.9%) were AmpC &ESBL co-producers by cefepime/ cefepime clavulanic E.test. To conclude it is important to know the prevalence of ESBL, AmpC producing and ESBL&AmpC co-producing organisms so that judicious use of antibiotics could be done and increase awareness about the need for routine detection of AmpC &ESBL in clinical isolates. CHROMagar™ ESBL media detect ESBL producers from clinical specimen and give rapid presumptive identification by means of colony colour at 24h with good sensitivity and specificity.

INTRODUCTION

Microorganisms responsible for urinary tract infection (UTI) such as *E.coli* and *Klebsiella* spp. have the ability to produce ESBL in large quantities. These enzymes are plasmid borne and confer multiple drug resistance, making urinary tract infection difficult to treat.^[1]

Enterobacteriaceae, especially *E. coli* and *Klebsiella* spp.-producing ESBLs such as SHV and TEM types, have been established since the 1980s as a major cause of hospital-acquired infections. However, during the late 1990s, several community-acquired pathogens that commonly cause urinary tract infections and diarrhea have also been found to be ESBL producers. These include *E. coli*, *Salmonella*, *Shigella* and *Vibrio Cholera*.^[2]

ESBLs are enzymes capable of conferring bacterial resistance to the penicillins, first, second- and third-generation cephalosporins, and aztreonam. They are not active against the cephamycins (cefoxitin and cefotetan), but are susceptible to β -lactamase inhibitors (clavulanic acid)^[3].

Genotypic methods based on enzyme assays, PCR and others are not suitable for routine clinical testing in most clinical diagnostic laboratories. The phenotypic confirmatory tests are highly sensitive and specific compared to genotypic confirmatory tests.^[4]

Phenotypic tests, which require a screening step followed by confirmation used to detect ESBL in most microbiology laboratories. The screening test is based on testing the organism for resistance to an indicator cephalosporin. Cefpodoxime is commonly used as it is hydrolysed by TEM, SHV, and CTX-M types, but other cephalosporins such as cefotaxime, ceftriaxone, and ceftazidime are also used. To confirm the presence of an ESBL, synergy between the indicator cephalosporin and clavulanic acid needs to be demonstrated (ESBLs are inhibited by clavulanic acid).^[5] E test is a convenient method for detection of ESBL by MIC reduction. Two different E test gradient formats have been in use based on reduction of ceftazidime or cefotaxime MICs by 3 two-fold dilutions in the presence of clavulanic acid and have been used successfully for ESBL detection^[6,7].

Various selective media have been proposed in order to assess the presence of ESBL producers in stool and urine samples. Examples of such media include Drigalski agar supplemented with cefotaxime, MacConkey agar supplemented with ceftazidime or nutrient agar supplemented with ceftazidime, vancomycin and amphotericin B. Chromogenic media were developed for isolation of ESBL from clinical specimens. CHROMagar™ ESBL media

allows the detection of ESBL-producing bacteria while inhibiting the growth of other bacteria. Identification of ESBL producers depend on colony colour after 24h incubation^[8].

The ESBL phenotypes have become more complex due to the production of multiple enzymes which include the inhibitor-resistant ESBL variants and plasmid-borne AmpC. AmpC is normally produced in low levels by many organisms and is not associated with resistance, but it can be produced at high levels and cause resistance to all beta lactams, except carbapenems and 4th generation cephalosporins. These enzymes have spread worldwide and their prevalence varies by the geographical area. Many clinical laboratories currently test *Escherichia coli* and *Klebsiella* spp. for production of ESBLs but do not attempt to detect plasmid mediated AmpC beta lactamases. These enzymes are typically associated with multiple antibiotic resistances, leaving a few therapeutic options^[9].

The present study was done to detect the prevalence of ESBL, AmpC producing and ESBL&AmpC co-producing strains of *E. coli* in urinary tract infections patients in Benha University Hospital and to evaluate the performance of CHROMagar™ ESBL media for rapid screening of ESBL producing *E. coli*.

MATERIALS AND METHODS:

This study was done in Microbiology and Immunology Department of Benha Faculty of Medicine from October 2012 to March 2013. Out of the 135 urine samples collected from patients suspected to have UTI, a total of 100 were culture positive on CLED, collected from 100 patients (56 males and 44 females) who were diagnosed as UTIs patients in Benha University hospital either from Urology Department or attending Urology Benha University outpatient clinic. Their ages ranged from 20 to 68 years. The selection of patients was done according to clinical data suspecting UTI. The diagnosis of UTIs in urine samples was based on the presence of 10^5 CFU of microorganisms per ml in urine culture on CLED^[10]. Also, presence of more than 5 pus cells per high power field in an unspun urine in male, and more than 10 pus cells in a female. High number of pus cells in urine, or pyuria usually indicates infection^[11].

All patients under study were subjected to:

Full history taking and clinical examination with specific stress on fever, urgency, frequency, dysuria, suprapubic tenderness and costovertebral angle pain and tenderness.

Bacteriological study include:

Samples : urine sample was collected from each patient in sterile and screw capped urine container and subjected to the following:

I- Urine analysis

II- Urine culture

- Urine culture on CLED agar, CHROMagar™ ESBL and MacConkey agar supplemented with 2 mg/liter ceftazidime (MCKC)
- Identification of bacterial isolates by colony colour & morphology, gram stain and biochemical reactions

III- ESBL detection in E. coli isolates by:

- 1- Phenotypic screening CLSI method, CHROMagar™ ESBL and MCKC
- 2- Phenotypic confirmation by E test

IV- AmpC beta-lactamase isolates were screened by ceftaxime resistance (zone < 19mm) then confirmed by AmpC disc test

V- AmpC beta-lactamase ESBL co-producers detection by ceftaxime & ceftaxime+ clavulanate E test.

Methods :

A- Urine sample collection:

Early morning and mid-stream urine is collected in a sterile container for bacteriological and microscopic examination □after □cleaning the periurethral area (meatus, vulva & folds). If collected from indwelling catheter: the wall of the catheter at the juncture with the drainage tube should be disinfected and sterile syringe should be used to obtain a urine specimen under aseptic conditions. Immediate processing of the samples after collection was done to avoid samples contamination.

Viable bacterial count was performed for urine samples using the pour plate method. Microscopic examination of centrifuged urine was performed for pus cell (WBC) count ^[10]

B- Urine culture

1. The urine samples were centrifuged; cultures were done from the deposit on CLED agar, all plates were incubated at 37°C for 48 hrs.
2. Culture on CHROMagar™ ESBL(CHROMagar, France) which was freshly prepared on the day of the study according to the manufacturer's instructions and poured into 90-mm-diameter petri dishes. Urine was directly inoculated onto a CHROMagar plate and streaked for colony isolation. The plates were incubated overnight at 35°C in ambient air and then examined for any growth.^[12]
- Culture on MacConkey agar supplemented with 2 mg/liter ceftazidime (MCKC). The plates were incubated overnight at 35°C in ambient air and then examined for any growth.
- Identification of bacterial isolates by colony morphology, gram stain and biochemical reactions^[13]

Interpretation of CHROMagar™ ESBL according to colony colour:

E.coli: dark pink to reddish colonies

Klebsiella, Enterobacter, Citrobacter spp.:Metallic blue colonies

Proteus: brown halo colonies

Non Resistant Other Gram(-) strains and gram(+) strains inhibited

C- Testing for the ESBL Production:

1. Phenotypic screening CLSI method for ESBL Production:

- E. coli isolates were screened for ESBL production by the disc diffusion method according to the CLSI guidelines^[7]. The following antibiotics were used; cefotaxime (30µg), cefpodoxime (30µg), ceftriaxone (30µg), ceftazidime (30µg), cefepime (30µg), Aztreonam (30µg). (Oxoid, UK). This screening method based on measuring the specific zone diameters for the antibiotic discs
- All the strains which showed a zone diameter of ≤ 17 mm for cefpodoxime, ≤ 22 mm for ceftazidime, ≤ 27 mm for aztreonam, ≤ 25 mm for ceftriaxone and ≤ 27 mm for cefotaxime were selected for checking the ESBL production as was recommended by CLSI M100-S21 (2010)^[7]. Every isolate that showed resistance to at least one of the screening agents was tested for ESBL

production. The use of more than one of these agents for screening improves the sensitivity of detection.

2. *Phenotypic confirmatory test by ESBL-E-test:*

The ESBL-E-Test strips were obtained from (AB biodisc, Solna, Sweden) ceftazidime/ceftazidime + clavulanic acid (TZ/TZL) and cefotaxime and cefotaxime + clavulanic acid (CT/CTL) in accordance with the manufacturer's instructions. One end of each strip contains a gradient concentration of either ceftazidime (TZ) (MIC range 0.5 to 32 µg/ml) or cefotaxime (CT) (MIC range of 0.25 µg to 16 µg). The other end of the strip with a gradient of ceftazidime plus a constant concentration of clavulanate TZ/TZL (0.064-4 µg/ml plus 4 µg/ml of clavulanic acid) or with a gradient of cefotaxime plus a constant concentration of clavulanate CT/CTL (0.25 µg- 16 µg plus 4 µg of clavulanic acid)[14]. After overnight growth, the organism was emulsified in saline solution to a turbidity of 0.5 McFarland standard. The suspension was spread on a Muller Hinton agar plate with a cotton swab. After the plates were dried for 15 min, the E-Test strips were placed on them, after incubation at 35°C for 18 hrs. The MIC was interpreted as the point of intersection of the inhibition ellipse with the edge of the test strip. After overnight growth, the organism was emulsified in saline solution to a turbidity of 0.5 McFarland standard. The suspension was spread on a Muller Hinton agar plate with a cotton swab. After the plates were dried for 15min, the E-Test strips were placed on them,after incubation at 35°C for 18 hrs. The MIC was interpreted as the point of intersection of the inhibition ellipse with the edge of the test:

1) ESBL positive:

- If $CT \geq 0.5$ and $CT/CTL \geq 8$ or $TZ \geq 1$ and $TZ/TZL \geq 8$.
- Presence of a phantom zone or ellipse deformation

2) ESBL Negative: If $CT < 0.5$ or $CT/CTL < 8$ and $TZ < 1$ or $TZ/TZL < 8$.

3) Non-determinable (ND): $CT > 16$ and $CTL > 1$ and $TZ > 32$ and $TZL > 4$. Strains showing non-determinable (ND) results with CT/CTL and TZ/TZL strips should be further tested using PM/PML strips for detection of AmpC beta-lactamase ESBL co-producers.

D- *AmpC beta-lactamase detection by AmpC disc test*^[16]

The test is based on use of Tris-EDTA to permeabilize a bacterial cell and release β-lactamases into the external environment. Cefoxitin (30µg). (Oxoid, UK) resistant *E.coli* isolates tested by AmpC disc test as following:

1. A lawn culture of a 0.5 McFarland's suspension of ATCC *E.coli* 25922 (bioMérieux) was prepared on a Mueller-Hinton agar plate. A 30 µg cefoxitin (fox) disc was placed on the inoculated surface of the agar. A sterile AmpC discs (i.e., filter paper discs containing Tris-EDTA) obtained from (Becton Dickinson, Sparks, MD) which was inoculated with several colonies of the test organism was placed beside the cefoxitin disc, almost touching it.
2. After an overnight incubation at 37°C, the plates were examined for either an indentation or a flattening of the zone of inhibition, which indicated the enzyme inactivation of cefoxitin (positive result), or an absence of distortion, which indicated no significant inactivation of cefoxitin (negative result).

E- AmpC beta-lactamase ESBL co-producers detection:

a) Cefepime/cefepime + clavulanic acid (PM/PML) strips ^[15]

All non-determinable ESBLs by TZ/TZL and CT/CTL E test were further tested using PM/PML strips for detection of AmpC beta-lactamase ESBL co-producers.

One end of each strip contains a gradient concentration of Cefepime (PM) (MIC range 0.25-16 µg/mL). The other end of the strip with a gradient of Cefepime plus a constant concentration of clavulanate PM/PML (0.064-4 µg/mL plus 4 µg/ml of clavulanic acid). The results were interpreted as positive:

- If the MIC ratio for PM/PML was ≥ 8 .
- Presence of a phantom zone, deformation or ellipse

Statistical analysis: Data were entered into a database using SPSS 13 for Windows (SPSS Inc., Chicago, IL). Sensitivity: the ability of the test to detect true positive cases and specificity: the ability of the test to detect true negative cases ^[17]

RESULTS :

The ages of studied patients ranged from 20 to 68 years. As regards the sex distribution among the studied patients out of 100 patients 56 were males and 44 were females. 45 were out-patients and 55 in-patients admitted in Urology department. Out of 55 in-patients 30 patients were catheterized and 25 were non catheterized. 105 isolates on CLED agar were obtained from

the studied 100 patients urine samples, while most samples yielded only single isolate, five samples yielded two isolates. The most common isolated microorganisms were *E.coli* 45 (42.9%) followed by *Klebsiella pneumoniae* 22 (21%) and the least was *Staphylococcus aureus* 2 (1.9%).

Out of 45 *E. coli* strains 24 (53.3%) ESBL producers were detected by CT/CTL and/ or TZ/TZL E. test (golden method for confirmation of ESBL according to CLSI) and 21 (46.7%) strains were non ESBL producers as shown in figure (1)

Table (1) shows that out of the 24 isolated ESBL producing *E.coli* strains 9 (37.5%) were detected in community acquired UTI either out-patients or in-patients admitted <48 h patients while 15 (62.5%) were detected in health care associated UTI patients who were admitted >48 h.

Table (2) shows that out of 45 *E. coli* isolates 16 (35.6%) yielded no growth on any selective media and 29 (64.4%) yielded growth on MCKC, while 27 (60%) yielded growth on CHROMagar™ ESBL media. In comparison with the E. test, sensitivity of the MCKC and CHROMagar™ ESBL media were 100%. While specificity were 80.8% and 87.5% respectively.

Preliminary screening test by CLSI method showed that resistance of *E coli* strains to Ceftazidime, Ceftriaxone, Aztereonam, Cefpodoxime and Cefepime were 35 (77.7%), 33(73.3%), 31(68.9%), 26 (57.8%), 26 (57.8%) respectively, so 35 strains (77.8%) of isolates were considered as preliminary producers of ESBLs. While preliminary producers of ESBLs from CHROMagar™ ESBL agar were 27 (60%) as shown in Table (3). 24 (100) confirmed ESBLs were detected by both CLSI screening method and CHROMagar™ ESBL agar. 11 were positive ESBLs by CLSI screening method and 3 positive ESBLs by CHROMagar™ ESBL agar, while all these strains were confirmed negative. Sensitivity of both CLSI screening method and CHROMagar™ ESBL agar were 100%. While high specificity showed by CHROMagar™ ESBL agar 100%, low specificity showed by CLSI screening method 70%.

Among the screen positive isolates cefoxitin resistance was noted in 41 (91.1%) isolates. All the ESBL producers (24), 14 AmpC producers and 4 AmpC &ESBL co-producers were cefoxitin resistant.

Table (4) shows that out of 45 isolated *E.coli* strains 14 (31.1%) were AmpC producers by AmpC test, 4 (8.9%) were AmpC &ESBL co-producers by cefepime/ cefepime clavulanic E.test, 24 (53.3%) were pure ESBL by TZ/TZL or CT/CTL E.test.

233 Table (1) Distribution of ESBL producing *E. coli* among community acquired and health
234 care associated UTI infections:

ESBL producing <i>E. coli</i> (n=24)	Community acquired UTI		Health care associated UTI	
	NO	%	NO	%
	9	37.5%	15	62.5%

235 Table (2) Identification of ESBL producer and non ESBL producer *E. coli* growth on
236 CHROMagar™ ESBL agar and MCKC agar

Results of growth	CHROMagar™ ESBL agar		MCKC	
	NO	%	NO	%
Growth	27	(60%)	29	64.4%
No growth	18	(40%)	16	35.6%
TOTAL	45	45	45	45
Specificity	87.5 %		80.8 %	
Sensitivity	100%		100%	
PPV	88.9 %		82.8 %	
NPV	100%		100.00 %	

237 Table (3) Comparison between CHROMagar™ ESBL agar and CLSI screening method to
238 screen ESBL producing *E. coli*:

	CHROMagar™ ESBL agar		CLSI screening method	
	NO	%	NO	%
Preliminary ESBL producers	27	60%	35	77.8%
Non ESBL producers	18	40%	10	22.2%
Total	45	100	45	100%

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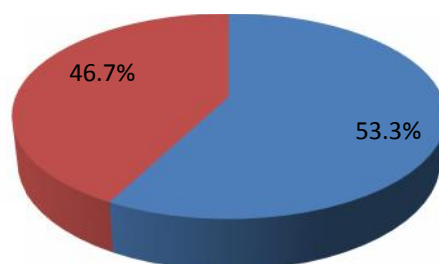
240 Table (4) Distribution of AmpC and AmpC &ESBL co-producers among *E. coli* isolates:

<i>E. coli</i> isolates n=45							
Amp C producers		Amp C &ESBL co-producers		Pure ESBL producers		Non Amp C &ESBL producers or co-producers	
NO	%	NO	%	NO	%	NO	%
14	31.1%	4	8.9%	24	53.3%	3	6.7%

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Figure (1) Distribution of ESBL producing *E. coli* among UTI patients

■ ESBL producing E.coli ■ Non ESBL producing E. coli



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244 Figure (2) Dark pink to reddish *E. coli* colonies isolated on CHROMagar™ ESBL media



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Figure (3) Positive AmpC test (indentation of the zone of inhibition around cefoxitin disc)



DISCUSSION:

Extended-spectrum β -lactamases (ESBLs) producing Gram-negative bacteria are large, rapidly evolving group of plasmid-mediated enzymes emerging pathogens. Clinicians, microbiologists, infection control practitioners and hospital epidemiologists are concerned about ESBL-producing bacteria because of the increasing incidence of such infections^[18]

In the present study out of 45 *E. coli* strains 24 (53.3%) ESBL producers were detected by E. test (golden method for confirmation of ESBL according to CLSI^[7]). This is in agreement with *Hasan et., al*^[19] who reported (57.4%) ESBL rate in uropathogenic *E.coli*. *Thabit et.,al*^[20] also reported 53% ESBL producing *E.coli* by E. test.

In our study out of the 24 isolated ESBL producing *E.coli* strains 9 (37.5%) were detected in community acquired UTI patients while 15 (62.5%) were detected in health care associated UTI patients. This was in agreement with *Thabit et.,al*^[20] Who reported that ESBL producing *E. coli* among community isolates was (39.47%) while among nosocomial isolates the rate was (70%). Our results are comparable to those obtained by *Bean et al.*^[21]. who reported a community-based ESBL prevalence to be 5.7% in London. The cause of the upsurge in community-acquired infections with ESBL-producing organisms is not yet clear, but associations with foodstuffs, animal consumption of antibiotics, and frequent patient contact with health care facilities need to be explored^[22].

Methods to detect ESBL-producing organisms from clinical specimens should have high sensitivity and high specificity combined with a short time to the reporting of results. In order to

identify ESBL-producing gram-negative bacilli from clinical samples more easily and reliably, selective media should ideally achieve the identification of the organisms and detection of ESBL in one step. At the least, it should decrease the workload and reduce the need of unnecessary confirmations.^[23]

In the present study out of 45 *E. coli* isolates 16 (35.6%) yielded no growth on any selective media and 29 (64.4%) yielded growth on MCKC, while 27 (60%) yielded growth on CHROMagar™ ESBL media. In comparison with the E. test, sensitivity of the MCKC and CHROMagar™ ESBL media were 100%. While specificity were 80.8% and 87.5% respectively. **Glupczynski et., al**^[24] reported sensitivity and specificity of MCKC 84% and 91% respectively. Regarding sensitivity and specificity of CHROMagar™ ESBL media **lagace-Wiens et., al**^[25] reported high sensitivity (99,2%) and Specificity (89%). Also **Sito et., al**^[26] reported sensitivity and specificity of CHROMagar™ ESBL media 100% and 93% respectively.

In our study preliminary screening test by CLSI screening method showed that 35 strains (77.8%) of isolates were considered as preliminary producers of ESBLs. While preliminary producers of ESBLs from CHROMagar™ ESBL agar were 27 (60%) as shown in Table (3). 24 (100) confirmed ESBLs were detected by both CLSI screening method and CHROMagar™ ESBL agar. 11 were positive ESBLs by CLSI screening method and 3 positive ESBLs by CHROMagar™ ESBL agar, while all these strains were confirmed negative. Sensitivity of both CLSI screening method and CHROMagar™ ESBL agar were 100%. While high specificity showed by CHROMagar™ ESBL agar 100%, low specificity showed by CLSI screening method 70%. Also **Manhas et.,al**^[27] reported sensitivity and specificity of CLSI screening method were 99.4% and 66.1% respectively. **Thabit et.,al**^[20] reported 76.5% potential producers of ESBLs by CLSI screening method while confirmed ESBLs 53% .

With the spread of AmpC and ESBL producing strains all over the world, it is necessary to know the prevalence of these strains in hospitals. Use of cefepime is more reliable to detect these strains because high AmpC production has little effect on cefepime activity. In this study out of 45 isolated *E.coli* strains 14 (31.1%) were AmpC producers by AmpC test, 3 (6.7%) were AmpC &ESBL co-producers by cefepime/ cefepime clavulanic E.test, 24 (53.3%) were pure ESBL by TZ/TZL or CT/CTL E.test. This was in agreement with **Singhal et.,al**^[28] who

reported AmpC enzyme production in 36% of *E.coli* isolates by AmpC test,also **Sinha et.,al**^[29] reported AmpC production in 24% and co-production of ESBL and AmpC enzymes in 8%. **Stürenburg et al**^[30]evaluated the performance of the cefepime clavulanate ESBL E test to detect AmpC &ESBL co-producers in an Enterobactriaceae strain collection. The ESBL E test was 98% sensitive with cefepime-clavulanate.

CONCLUSION:

- 1- It is important to know the prevalence of ESBL, AmpC producing and ESBL&AmpC co-producing organisms so that judicious use of antibiotics could be done.
- 2- CHROMagar™ ESBL media detect ESBL producers from clinical specimen and give rapid presumptive identification by means of colony colour at 24h with good sensitivity and specificity.

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