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ORIGINAL ARTICLE

Detection of ESBL Producing Bacteria in Cases of Urinary Tract Infection in Pediatric Department at Benha University Hospital

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Children UTI, ESBL, SHV, CTXm , Multiplex PCR

ABSTRACT

Background: *Extended-spectrum beta-lactamases (ESBLs) are rapidly evolving group of beta-lactamase enzymes commonly produced by the Gram negative bacteria. Objectives: This work aimed to detect the bacterial pathogens that cause urinary tract infection and to detect ESBL producing bacteria among them in Pediatric Department at Benha University Hospital.* ***Methodology:*** *This study was conducted on 118 children patients suffering from UTI collected from Outpatient and Inpatient of pediatric department at Benha University during the period from January 2015 to the end of July 2015. They were 77 girls and 41 boys and their ages ranged from 1 to 13 years.118 Urine samples were subjected to isolation and identification of causative organisms, screened for ESBL production by the pattern of antimicrobial susceptibilities. Phenotypic identification for ESBL production was confirmed by double disc diffusion test, bla SHV and bla CTX-M genes in ESBL, producing isolates were detected by using multiplex PCR.* ***Results:*** *This study showed that the most common cause of children UTI was E.coli (55.1%) followed by klebsiella pneumoniae (21.2%). Thirty seven (31.4%) of 118 bacterial isolates that tested by double disc confirmatory test were ESBL positive. Among the ESBL producing bacteria, 16 out of 37 (43.24%) have CTX-m and SHV genes together while 3 out of 37 (8.1%) have SHV gene only.* ***Conclusion:*** *Our findings showed that majority of ESBL isolates were positive for both CTXm and SHV genes (43.24%) but SHV gene alone was positive only in (8.1%) of isolates, So studies should be done in different regions of Egypt to find the common ESBL genes present in that geographical area for epidemiological purposes. Also ESBL producing isolates have been increasingly recognized in hospitals in Egypt and are associated with multiple drug resistance. Thus, ESBL producing organisms should be identified quickly so that appropriate antibiotic usage and infection control measures can be implemented.*

INTRODUCTION

Urinary tract infection is a serious health problem affecting children each year. Infections of the urinary tract are the second most common type of infection after the infection of the respiratory tract [1].Acute UTIs are relatively common in children. By seven years of age, 8 % of girls and 2 % of boys will have at least one episode [2]. Baseline abnormalities of the urogenital tract have been reported in up to 3.2 % of healthy, screened infants[3].Common uropathogens are *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter, Citrobacter Freundi*, *Staphylococus saprophyticus, and Enterococci* [4] .

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In older kids, UTIs may cause obvious symptoms such as burning or pain with urination. In infants and young children, UTIs may be harder to detect because symptoms are less specific. In fact, fever is sometimes the only sign [5].Gram-negative bacteria show high level of resistance to commonly used empirical β-lactam antibiotics(Ampicillin, Augmentin and Cefaclor), fluoroquinolones (Ciprofloxacin and Ofloxacin) and co-trimoxazole [6].

Beta-lactamases are enzymes that open the beta-lactam ring, inactivating the antibiotic. According to the type of their substrates , they are divided into four functional groups: penicillinase, extended spectrum beta-lactamase (ESBL), carbapenemase and cephalosporinase type AmpC [7] .ESBL is found in certain genera of the Enterobacteriaceae family including *Escherichia coli* and *Klebsiella pneumoniae* and other bacteria like *Haemophilus influenza and Pseudomonas aeruginosa*[8] .ESBL arise mainly due to mutation in β-lactamases encoded by the bla SHV, blaTEM, and bla CTX-M genes. More than 300 different ESBL variants

have been described [9]. Though TEM and SHV variants are the most common ESBLs. During the past decade, strains expressing CTX-M ESBLs have begun to emerge in many countries and are now the most frequent non-TEM, non-SHV ESBL type[10].

CTX-M b-lactamases are characterized by selective hydrolysis of cefotaxime rather than ceftazidime, though some CTX-M types, such as CTX-M-15, may actually hydrolyse ceftazidime [11]. The aim of the present study is to detect the pathogens that cause urinary tract infection and to detect ESBL producing bacteria among them in Pediatric Department at Benha University Hospital .

METHODOLOGY

This work was carried out in Microbiology and Immunology Department, Faculty of Medicine, Benha university from January 2015 to the end of July 2015.

1. **Subjects:** The study was conducted on 118 patients suffering from UTI collected from Outpatient and Inpatient from Pediatric Department, Benha University Hospital. Full medical history was taken included the associated disease. They were 77 girls and 41 boys and their ages ranged from 1 to 13 years. A written informed consent (in Arabic language) was obtained from the patients parents before Participation.
2. **Samples**
   - In young children, the application of an adhesive, sealed, sterile collection bag after disinfection of the skin of the genitalia by diluted alcohol (70%)was done. In those who were toilet trained. The urine samples were midstream samples[12].

The collected urine samples were subjected for the followings [13]:

a- Urine examination

Each specimen was divided into 2 parts:

- *Uncentrifugated part* to perform viable count. After viable count performing and incubation, the urine culture samples were classified as negative, positive, or contaminated. When polymorphic bacterial growth (two or more bacterial species growth in one plate) was observed, the samples were classified as contaminated (exclusion criteria). The urine cultures were considered as negative when bacterial growth was lower than 10^3 CFU/mL (exclusion criteria).. When monomorphic bacterial growth was higher than 10^5 CFU/mL, the culture was classified as positive (inclusion criteria).
- *Centrifugated part:* The sediment was used to perform wet film and examined microscopically for pus cells to make stained film and culture .

b- Isolation and identification:

The sediment was directly cultured on nutrient agar, blood agar, MacConkey's medium and CLED agar without delay. The plates were incubated aerobically at 37ºC for up to 48 hours. Grown colonies were identified by biochemical reaction tests[14].

c- Antibiotic susceptibility testing :

This was done by using Muller Hinton agar *(Oxoid)* and antibiotic discs *((Oxoid)* including ampicillin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), cefotaxime (30 µg) and ceftazidime (30 µg). After over night incubation at 37˚C, inhibition zone diameters were read[15].The results of a disc diffusion test are interpreted by comparing the measured zone diameter with the interpretive criteria recommended by CLSI guidelines[16].

d- Phenotypic confirmation tests for ESβL:

Ceftazidime (30 ug) and cefotaxime (30 ug) discs were used alone and in combination with Clavulanic acid (10ug) for phenotypic confirmation of the presence of ESBLs .If the increase in zone diameter ≥ 5 mm for either of the cephalosporin discs and their respective cephalosporin / clavulanic acid disc was interpreted as ESBL Producer [17].

e- Genotypic detection of SHV and C-TXM genes by using Multiplex PCR:

Multiplex PCR testing of all ESBL isolates for SHV & CTX-m genes was done according to Ola et al [17]. Two primer pairs (Biosearch technologies, USA) were used.

DNA extraction:

Total DNAs of the different bacterial isolates were extracted by the DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit, EU Lithuania, #K0721) according to manufacturer instructions. The extracted DNA was then stored at -20°C until further processing.

DNA amplification:

Amplification was done using Maxima Hot Start PCR Master Mix #K1051 (Thermo Scientific, EU Lithuania). The PCR mix contained 25ul of PCR master Mix, 2.5ul of each forward primer, 2.5ul of each reverse primer, 5ul of the template DNA and the amount completed with nuclease free water(10ul) to reach a final volume of 50ul. *Biometra ,Germany* thermal cycler was used for amplification according to the following program: initial denaturation at 95 ºC for 4mins, 45 cycles of denaturation at 95 ºC for 30 s, annealing at 46 ºC for 30 s and extension at 72 ºC for 45 s, followed by final extension at 72 ºC for 10 mins.

Sequence of primer for bla SHV gene were:

5'ATT TGT CGC TTCTTT ACT CGC-3' (*Biosearch technologies*, USA, SS339883-09) as a forward primer and 5'- TTT ATGGCG TTA CCT TTG ACC-3' (*Biosearch technologies*, USA, SS339883-10) as a reverse primer.

Sequence of primer for bla CTX-m gene were:

5'-ATGTGC AGY ACC AGT AAR GT-3'(*Biosearchtechnologies*, USA, SS339883-80) as a forward primer and5'-TGG GTR AAR TAR GTS ACC

AGA-3' (*Biosearch technologies*,USA, SS339883-32) as a reverse primer.

DNA detection by agarose gel electrophoresis:

The detection of(1018bp) and (544bp) amplified products of SHV and CTXm genes respectively by agarose gel electrophoresis was carried out according to[17]. 10µl of each amplified DNA & 100bpladder (molecular weight marker) were separated on 2% agarose gel containing 0.3 mg/ml of ethidium bromide. The bands were visualized using UV transilluminator (312 nm,Biometra ,Germany), photographed & analyzed.

3- Statistical analysis:

Data were recorded and analyzed using STATA/SE version 11.2 for Windows (STATA corporation, College Station, Texas).

RESULTS

This study was conducted on 118 children patients who had symptoms suggestive of UTI. They were either Outpatient Pediatric children (72%) or Inpatient Pediatric cases (28%) .They were 77 (65.25%) girls and 41(34.75%) boys. Their ages ranged from 9-13 years as shown in table 1 .

Table 1: Distribution of the studied group according to age:

Age (years)	ESBL (No.=37)		Non-ESBL (No.=81)		Total (No.=118)		Test	P
	No.	%	No.	%	No.	%		
<3	5	13.51	12	14.81	17	14.41	χ^2= 3.55	0.31
3 to <6	14	37.84	18	22.22	32	27.12		
6 to <9	10	27.03	24	29.63	34	28.81		
9 to 13	8	21.62	27	33.33	35	29.66		
Mean ± SD; (range)	5.96±2.93; (1-12.1)		7.02±3.28; (1.4-13)		6.69±3.20; (1-13)		t= 1.70	0.09

There was a significant difference in the proportion of ESBL positive and negative patients between inpatients and outpatients (P=0.01). The percentage of ESBL positive patients was higher among inpatients compared to ESBL negative patients (43.24% *vs*. 20.99% respectively) As shown in fig. 1.

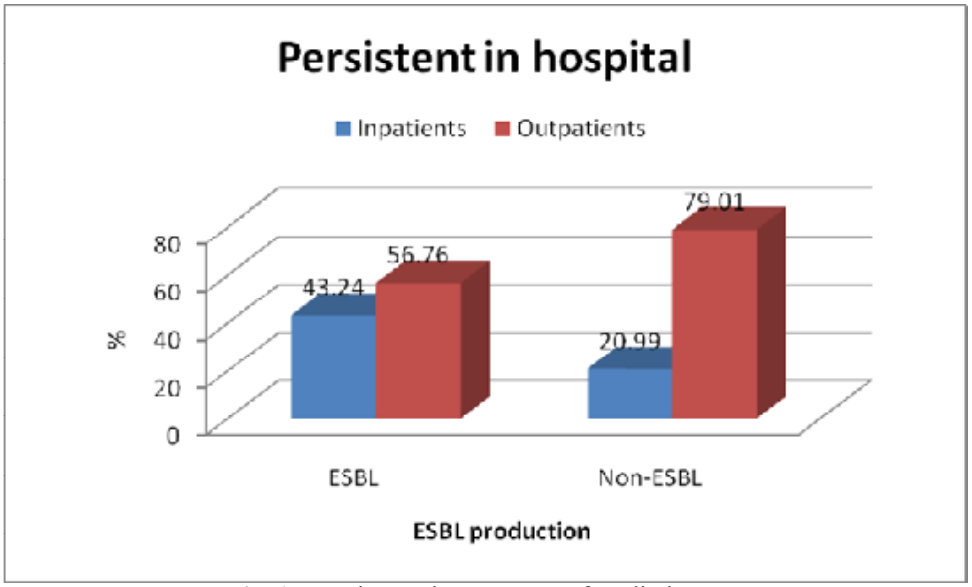

Fig. 1: Number and percentage of studied cases.

Urinary tract symptoms were present in 95 cases (80.5%) and absent in 23 cases (19.5%). The symptoms were Fever, convulsions, vomiting, Diarrhea, haematuria, dysuria and Pain during urination. There was a significant difference in the proportion of presence and absence of symptoms according to ESBL positive and negative patients (P=0.03). The proportion of present symptoms was higher among ESBL positive patients compared to absence symptoms (91.89 % *vs*. 8.11% respectively) as shown in table 2.

Table 2: Distribution of the studied group according to urinary tract symptoms

Urinary symptoms	ESBL (No.=37)		Non-ESBL (No.=81)		Total (No.=118)		χ^2	P
	No.	%	No.	%	No.	%		
Absent	3	8.11	20	24.69	23	19.49	4.45	0.03 (S)
Present	34	91.89	61	75.31	95	80.51		

S: significant (P<0.05)

The most common associated disease with UTI in children was the HAV then renal stone . There was a significant difference in the percentage of ESBL positive and negative patients in case of non associated disease (P=0.03). The percentage of ESBL negative patients was higher than ESBL positive patients (40.74% *vs*. 13.51% respectively).The result is presented in table 3.

Table 3: Classification of the studied group according to different associated diseases

Associated disease	ESBL (No.=37)		Non-ESBL (No.=81)		Total (No.=118)		Z	P
	No.	%	No.	%	No.	%		
No associated disease	5	13.51	33	40.74	38	32.2	2.94	0.003(S)
Stone	5	13.51	11	13.58	16	13.56	0.01	0.99
Renal failure	1	2.7	5	6.17	6	5.08	0.80	0.42
Renal atrophy	3	8.11	2	2.47	5	4.24	1.41	0.16
Nephrotic syndrome	0	0.0	2	2.47	2	1.69	0.96	0.33
Scrotal tumor	1	2.7	1	1.23	2	1.69	0.57	0.56
Uretar dilatation operation	1	2.7	0	0.0	1	0.85	1.48	0.14
HAV	12	32.43	15	18.52	27	22.88	1.67	0.09
HCV	4	10.81	4	4.94	8	6.78	1.18	0.24
HBV	0	0.0	1	1.23	1	0.85	0.68	0.50
Diabetes	5	13.51	7	8.64	12	10.17	0.81	0.42

The most common cause of children UTI were *E.coli 66*(55.93%) followed by *klebsiella pneumoniae* 25 (21.19 %), *Pseudomonas aeruginosa* 13 (11.02%) , *Proteus mirabilis* 8 (6.78%), *Staph aureus* 2 (1.69%) and *Citrobacter freundi* 4(3.39%).There was a high significant difference in the proportion of ESBL positive and negative patients according to the isolated organisms *E.coli* and *Klebsiella pneumoniae* (P<0.001). The percentage of ESBL positive patients was higher among *Klebsiella pneumonia*e compared to ESBL negative patients (40.54% *vs*. 12.35% respectively) unlike in case of *E.coli* , The percentage of ESBL negative patients was higher compared to ESBL positive patients(66.67% *vs*. 32.43% respectively). The results are shown in table 4.

Table 4:Classification of the studied group according to isolated organism :

Isolated organism	ESBL (No.=37)		Non-ESBL (No.=81)		Total (No.=118)		Z	P
	No.	%	No.	%	No.	%		
E.coli	12	32.43	54	66.67	66	55.93	3.47	<0.001 (HS)
Klebsiella pneumoniae	15	40.54	10	12.35	25	21.19	3.47	<0.001 (HS)
Pseudomonas aeruginosa	5	13.51	8	9.88	13	11.02	0.58	0.56
Proteus mirabilis	3	8.11	5	6.17	8	6.78	0.39	0.70
Staph aureus	0	0.0	2	2.47	2	1.69	0.96	0.33
Citrobacter freundi	2	5.4	2	2.47	4	3.39	0.82	0.41

According to disk diffusion antibiotic sensitivity tests, we found that 20 (30.3) % of *E.coli* isolates ,19 (76%) of *klebsiella pneumoniae* , 8 (61.5%) of *Pseudomonas aeruginosa*, 5 (62.5%) of *Proteus mirabilis* and 2 (50%) of *Citrobacter freundi* were suspected to be ESBL producers.

By double disc confirmatory test we found that the *klebsiella pneumoniae* isolates were the highest ESBL positive among others as shown in table 5 and Fig 2.

Table 5: The Number and percentage of ESBL producing organisms by phenotypic double disc confirmatory test(DDT):

Organism		(+ve ESBL)	
	NO	NO	%
E.coli	20	12	(60%)
Klebsiella pneumoniae	19	15	(79%)
Proteus mirabilis	5	3	(60%)
Pseudomonas aeruginosa	8	5	(62.5%)
Citrobacter freundi	2	2	(100%)
Total	54	37	(68.5%)

This table shows that (68.5%) of bacterial isolates that tested by double disc confirmatory test were ESBL producing.

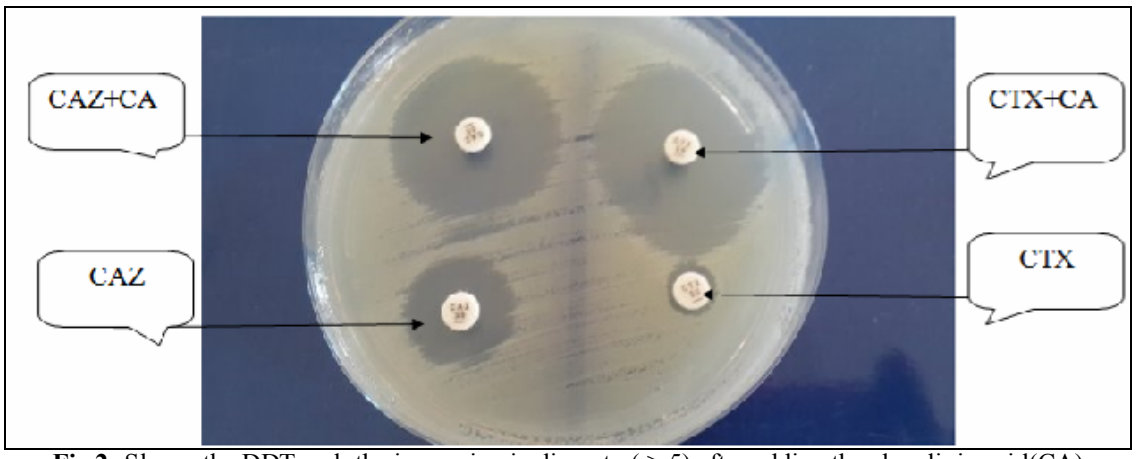

Fig 2: Shows the DDT and the increasing in diameter(≥ 5) after adding the clavulinic acid(CA) to the two antibiotics (ceftazidime(CAZ) and cefotaxime (CTX) (30 ug))

The percentage of of Ctx-m gene+ SHV gene was higher in ESBL positive isolates compared to SHV gene alone with high significance (P< 0.001) (43.24% *vs*. 8.1% respectively) .The results are shown in table 6.

Table 6: The Number and percentage of CTX-m and SHV genes in ESBL positive organisms by genotypic PCR technique

Organism		Ctx-m gene+ SHV gene		SHV gene alone		Z	P
	No.	No.	%	No.	%		
E.coli	12	5	41.7	1	8.33	4.18	<0.001(HS)
Klebsiella pneumonia	15	4	26.7	1	6.7	3.10	0.002(S)
Proteus mirabilis	3	2	66.7	0	0.0	-	-
Pseudomonas aeruginosa	5	3	60.0	1	20.0	2.24	0.02(S)
Citrobacter freundi	2	2	100.0	0	0.0	-	-
Total	37	16	43.24	3	8.1	7.83	<0.001(HS)

Figure 3 show a high significant difference in the proportion of (Ctx-m gene+ SHV gene) and (SHV gene only) according to PCR of ESBL (P<0.001)

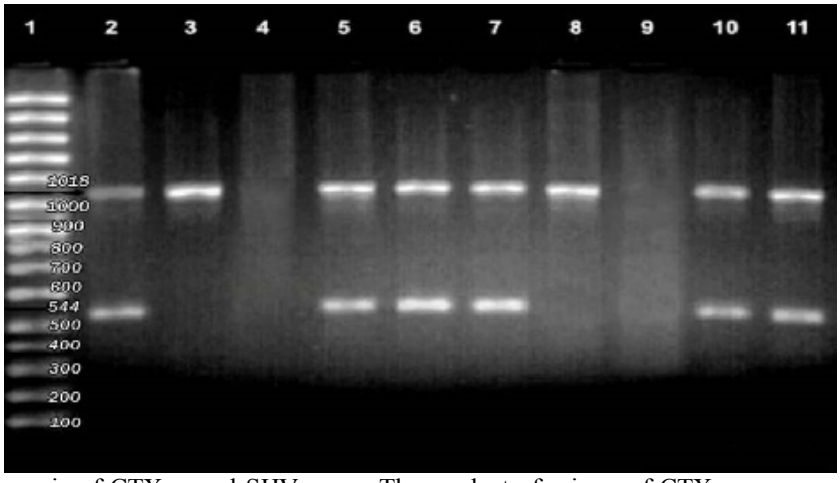

Fig. 3: Gel electrophoresis of CTX-m and SHV genes .The product of primer of CTXm gene was seen as band at 544 bpfragments and the product of primer of SHV gene was seen as band at 1018 bp fragments.
(1) is a ladder. Lanes (3,8) show one band with molecular weight 1018 bp (*SHV* gene) .lanes (2,5,6,7,10,11) show a bands with molecular weight 544 And 1018 bp (CTX-m And SHV genes). Lanes (4, 9) are negative .

DISCUSSION

Urinary tract infection is a serious health problem affecting children. Infections of the urinary tract are the second most common type of infection after the infection of the respiratory system[1]. ESBL is found in certain genera of the Enterobacteriaceae family including *Escherichia coli* and *Klebsiella pneumoniae* and other bacteria like *Haemophilus influenza* and *Pseudomonas aeruginosa* [8].

According to age distribution of our cases, the majority of UTI cases (29.66%) were between 9-13 years. In other studies it was (39.2%) in 3-*6 years*.

As regards sex of the UTI children patients, our study shows that girls are more susceptible to UTI than boys due to the differences in anatomy of their various tract . In girls, the moist periurethral and vaginal areas promote the growth of uropathogens. The shorter urethral length increases the chance for ascending infection into the urinary tract, which is consistent with Shah et al [18] .

In our study There was a statistically significant difference between Inpatients and Outpatients (P=0.01). The proportion of ESBL positive patients was higher among inpatients compared to ESBL negative patients (43.24% *vs*. 20.99% respectively), this means that the staying in hospital makes the patient more susceptible to infection with ESBL bacteria, which is in agreement with Michael et al [21] who found that the length of hospital stay prior to the urine sampling increase the rate of infection in comparison with Outpatients ($p<0.001$).

Also Ola et al[17] found that (40%)of ESBL isolates were obtained from outpatients and (60%) were from inpatients.

In our study urinary symptoms like (fever , crying with micturation and burning or pain with urination) were present in 80.5% of UTI . Fever and dysuria were the most common presenting symptoms in the present study .There was a statistically significant difference (P=0.03) as the percent of symptoms was higher among ESBL positive patients compared to absent symptoms (91.89 % *vs*. 8.11% respectively). Our study is consistent with John et al [22] who found that presenting symptoms, including anorexia, flank pain, diarrhea, vomiting, abdominal pain, hematuria, and decreased food intake, were noticed to be more common in children with ESBL UTI (95% vs. 79%, $p<0.05$) than non ESBL UTI.

While Shaw et al [24] found that urinary symptoms was present in 8.6% in the study done on 2411infant to establish prevalence rates of urinary tract infection (UTI) in febrile infants. Also Brein et al [25] found that specific urinary symptoms in 37% of UTI cases in his study.

The percentage of ESBL positive patients was higher among antimicrobial intake cases compared to ESBL negative patients (54.05% *vs*. 28.4% respectively), this can be explained by indiscriminate use of antimicrobial agent will lead to development of multiple drug resistant strains including ESBL producing bacteria.

Our study is in agreement with John et al [22] who found that Children with ESBL UTI were on antimicrobial therapy (21% vs 6%, $p<0.05$) compared to those with non-ESBL UTI.

In our study we found that *E.coli* was the most common isolated organism (55.1%) of cases followed by *klebseilla pneumoniae* (22.2%). This is in agreement with Mahmut et al[18] who isolated *Escherichia coli*

(64.5%), *Klebsiella pneumoniae* (12.1%) and *Proteus mirabilis* (8.4%) from UTI. Also Shah et al [18] isolated *E. coli* from UTI in 82.7% of patients, followed by *enterococcus spp*, *staphylococcus spp*, and then *Proteus mirabilis Klebsiella pneumonia*e and *Streptococci* . Alka et al [26] isolated *E.coli* from (44.96%) of cases, followed by *Enterobacter spp* (17.83%) and *Klebsiella spp* (14.72%).

Our study showed that *E.coli* was mostly sensitive to Amikacin (54.55%). The percent of ESBL negative strains was higher in sensitivity to ceftazidime compared to ESBL positive strains (50.0% *vs*. 25.0% respectively).

klebseilla pneumoniae is more sensitive to cefotaxime in (56%) of cases and more resistant to gentamycin (34.78%). This result agrees with Eman [27] .

Pseudomonas aeruginosa is mostly sensitive to ciprofloxacin (69.23%) and more resistant to ampicillin (84.62 %). This result agrees with Alka et al [26] .

According to the double disk diffusion method which was used as a phenotypic confirmatory test for ESBL detection, results of our study revealed That(68.5%) of the isolates were ESβL producers. These results are in partial agreement with Varun et al [28] who reported that (64.10%) of the Gram negative bacilli causing infections were ESβL producers.

In the present study ESβL were detected among 5 different species of *Enterobacteriaceae* and *pseudomonas* spp. *Klebsiella* spp was the most common ESβL-producing species constituting (40.5%) of ESβL gram negative bacilli, followed by *E.coli* (32.4%),then *Pseudomonas aeruginosa* (13.5).

Shilan and Fattma [23] showed that *E.coli* was the most common ESBL producer (66%),followed by *klebsiella spp* (15.5%) , then *proteus mirabilis* (7%)

In our study we used the PCR technique to search for the presence of SHV and CTX-m genes in ESBL positive isolates, were reported the two genes in(43.24%) of cases while (8.1%) isolates have SHV gene only . These results are in agreement with Mohammad et al [14] who reported that SHV gene was found in (44%) of cases and CTX-m in (28%) of cases only.

Our results showed that the most ESBL positive strain by phenotypic method was *klebseilla pneumoniae* while by Genotypic method was *E.coli* and this can be explained by the probapility of existence of ESBL encoded genes other than CTM and SHV.

CONCLUSION

- Our findings showed that the majority of ESBL positive isolates possess both CTX-m and SHV genes while a minority possess SHV gene alone, so studies are needed to detect the common ESBL genes in different geographical area for epidemiological purposes
- ESBL producing isolates have been increasingly recognized in hospitals and are associated with multiple drug resistance. Thus, ESBL producing organisms should be identified quickly so that appropriate antibiotic usage and infection control measures can be implemented.

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