Molecular detection of Extended Spectrum β-lactamases (ESBLs) genes in E.coli isolated from urine specimens

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ABSTRACT
The aim of this study was to detect and characterizing ESBLs producing E.coli isolated from clinical specimens from Wad Alabass specialized, Sennar state in the period between March 2011 to June 2012. A total 500 urine samples were collected and cultured on MacConkey agar and blood agar and proceed to identify UTI causative bacteria, by growth criteria on media, Grams stain and routine biochemical tests. 332(68%) samples gave a significant growth, the Gram-negative bacilli was 67%(232) of it , 93(28%) Gram-positive and 7 (2%) was yeast cells. Out 232(67%) Gram-negative bacteria 133 (40%) was E.coli ,33(10%) K.pneumoniae ,39(12%) Proteus spp, and 27(8%) Pseudomonas aeruginosa. ESBL production tests done For all E.coli isolates ,initially screened by cefotaxime and ceftazidime, where 62(46.6%) isolates gave positive result by both, then confirmed by combination test by using cefotaxime and ceftazidime with and without clavulanic acid, 48/62(77.4%) isolates gave positive result by both. The PCR was done For all E.coli isolates by using SHV, TEM and CTX-M primers, CTX-M gene was the most predominant one 30/46(65.22%) followed by TEM gene 13/46(28.26%) and the least one was SHV gene 3/46(6.52) genes.

Keywords: ESBL; Escherichia coli; CTX-M; TEM; SHV genes.

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1-INTRODUCTION
Several studies conducted in the world have reported a steady increase of the resistance rate of uropathogens to commonly prescribed antibiotics (amoxicillin, trimethoprim-sulfamethoxazole), reducing therapeutic possibilities (Gupta et al.,1999: Kahlmeter., 2003 ). The new broad-spectrum antibiotics such as ephalosporins used in treatment of bacterial infections has led to the production of a new class of broad-spectrum enzymes called betalactamase (Tenover, et al.,2003). Escherichia coli (E. coli) and Klebsiella pneumoniae are the frequently causes of nosocomial and community-associated infections such as urinary
tract infections, extended-spectrum cephalosporins are frequently used for treatment of infections by these species, resistance rates to these antimicrobial agents have also been increasing. (Murray et al., 2007). Therefore, the choice of antimicrobial agent to properly treat ESBL producers requires accurate identification. TEM-1, TEM -2 and SHV-1 β-lactamases that are capable of hydrolyzing penicillins but not cephalosporins. When certain mutations leading to substitutions of key amino acid residues are introduced, their kinetic properties are altered to allow for significant hydrolysis of cephalosporins, especially ceftazidime, and monobactam. These TEM- and SHV-derived ESBLs were the commonly observed ESBL types in E. coli throughout the 1980s and 1990s (Bradford., 2001). On the other hand, reports on a newer group of ESBLs, coined CTX-M for their preferential hydrolysis of cefotaxime over ceftazidime, started to emerge in E. coli in the late 1990s. (Paterson et al., 2005). Currently, five groups of CTX-M-type ESBLs have been identified: CTX-M-1, -2, -8, -9 and -25 groups. CTX-M-14 (belonging to the CTX-M-9 group) and CTX-M-15 (belonging to the CTX-M-1 group) are particularly associated with community-acquired isolates (Humeniuk et al., 2002). The ESBL producing E. coli can be detected by either phenotypic or genotypic methods, the genotypic methods seem to be necessary for accurate identification of such resistant strains. The aim of this study was to detect and characterize ESBL producing E. coli isolated from people with urinary tract infection.

2-MATERIALS AND METHODS

Study design and setting
This was a cross-sectional study conducted at Wad Alabass hospital which located in Sennar state (central of Sudan), in the period between March 2011 to June 2012.

Bacterial isolates
A total of 500 urine samples were obtained from patients attended to Wad Alabass hospital and plated on MacConkey agar and Blood agar medium, agar incubated at 37°C for 24 hours. A growth of $>10^5$ colony forming units per mL of one type of organism was considered as significant bacteriuria. Identification of E. coli was done by observing colonial morphology on MacConkey agar and Blood agar, colonies were further identified using standard biochemical tests (MacFaddin., 1980). The majority of growth was E. coli 133/332 (40%).
Antimicrobial susceptibility of *E. coli* isolates

Resistant to cefotaxime (30μg) and ceftazidime (30μg) antibiotics (Himedia, India) by Kirby-Bauer diffusion method according to Clinical Laboratory Standard Institute (CLSI) guidelines, (Podschun and Ullmann., 1998).

**Phenotype of ESBLs among *E. coli* isolates**

To distinguish the isolates resistant to any of the third generation of cephalosporins, ESBL production was tested by the MAST combined disk method (MAST Chemical Co, England). The bacterial suspension was prepared by agar dilution method matching the 0.5 McFarland standards. Two sets of disks of the following antibiotics were used in this study: ceftazidime (30μg), ceftazidime (30μg) plus clavulanic acid (10μg) and cefotaxime (30μg), cefotaxime (30 μg) plus clavulanic acid (10μg). (Wayne PA., 2006). Muller Hinton agar was inoculated with the bacterial suspension, and ESBL detection disks were placed on the surface of agar. Diameter of inhibition zone was measured after 18-24 hours of incubation at 37°C. A difference of ≥ 5 mm between the zone diameters of either of the Cephalosporin disk and their respective Cephalosporin/clavulanate disk is taken to be phenotypic confirmation of ESBLs production, (Andrea, *et al*., 2005). *E. coli* ATCC 25922 was used as control strains (Wayne, PA; 2006).

**Molecular analysis**

The plasmid DNA was extracted using phenol-chloroform-isoamylalcohol method(Baker., *et al* 1998). The concentration of extracted DNA was red using the spectrophotometer (Primer, 125v, 500 mA, UK). SHV, TEM, and CTX-M beta-lactamase genes were detected by PCR. PCRs were carried out using thermal cycler (CONVERGYS® td peltier thermal cycle, Germany). In a total volume of 25 μl containing 10 pmol of each three pair of primers (Metabion, GERMANY), 4 µl FIREPOL® Master mix (Solis BioDyne, Tartu, Estonia), 0.6 primer forward primer, 0.6 primer reverse primer (Table 1), 2µl plasmid DNA and 17.8 µl deionized sterile water. The PCR mixture was subjected to initial denaturation step at 94°C for 5-min, followed by 30 cycles of denaturation at 94°C for 45 s, primer annealing at 55°C for 45 seconds, followed by step of elongation at 72°C for 60 seconds and the final elongation at 72°C for 5 min. (Cao *et al*., 2002). PCR products were analyzed by electrophoresis in a 2% agarose gel in TBE 1X, containing 0.5 μg/mL ethidium bromide at 120V for 40 min. Bands were visualized under U.V transilluminater (Uvite –UK).
Table 1. Primers used for amplification of SHV, CTX-M and TEM genes

<table>
<thead>
<tr>
<th>Gene detected</th>
<th>Primer sequence (5’→3’)</th>
<th>Product size</th>
<th>Annealing Temp.</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>blatem</td>
<td>F: TCG GGG AAA TGT GCG CG R: TGC TTA ATC AGT GAG GCA CC</td>
<td>971 bp</td>
<td>55°C</td>
<td>(Cao et al., 2002)</td>
</tr>
<tr>
<td>blasvh</td>
<td>F: 5-GGTTATGCGTTATATCCG-3 R: 5-TTACCGGTGCCAGTCTC-3</td>
<td>850 bp</td>
<td>55°C</td>
<td>(Rasheed et al., 2002)</td>
</tr>
<tr>
<td>blactx-m</td>
<td>F: SCS ATG TGC AGY ACC AGT AA R: CCG CRA TAT GRT TGG TGG TG</td>
<td>550 bp</td>
<td>55°C</td>
<td>(Cao et al., 2002)</td>
</tr>
</tbody>
</table>

Statistical analysis
The data was recorded and analyzed. Statistical analysis was performed using SPSS statistical software, version 16.0 and the results was presented in a form of tables and figures.

3-RESULTS
Five hundred patients were enrolled at this study, were 348(70%) females and 152(30%) males.

Bacterial isolates
Out 500 urine samples 332(68%) samples gave a significant growth. *E. coli* [133 (40%)] was the most predominant organism isolated, as shown in the figure 1 bellow;

Figure (1) The distribution and percentage of bacterial growth
Detection of ESBLs

Of 133 E. coli isolates 62 (46.6%) isolates show resistant to cefotaxime and ceftazidime [60 (45%) isolates showed an inhibition zone of ≤ 27 mm for cefotaxime and 51 (38%) isolates showed an inhibition zone of ≤ 22 mm for ceftazidime], and when confirmed by Cephalosporin/clavulanic acid combination discs 48/62 (77.4%) isolates gave positive result, 39 (62.9%) showed a clavulanate effect with both CAZ and CTX, 2 (3.2%) showed a clavulanate effect with CAZ only, and 7 (11.2%) showed a clavulanate effect with CTX only), (See Figure 2 and 3). With a sensitivity of 74.1% (46/62) for cefotaxime and it is combination 78.4% (40/51) for ceftazidime and it is combination, as compared with screening tests. There were 4 isolates shows resistant even to clavulanate combination discs.

Figure (2). The phenotypic confirmatory test with cefotaxime (CTX) 30 μg on Muller Hinton agar with and Z).

Figure (3). The phenotypic confirmatory test with ceftazidime (CAZ) 30 μg on Muller Hinton agar ,

Genotypic detection of ESBLs

Of 133 E. coli isolates that were run by PCR machine only 46 (34.5%) isolates shows positive result, 30/46 (65.2%) CTX-M genes, 13/46 (28.3%) TEM genes and 3/46 (6.5%) SHV genes (See Figures 4, 5 and 6). While 2 isolates included two genes TEM and CTX-M
(See Table 2). Out 48 isolates that gave positive result by phenotypic tests there was only 42/48 (87.5%) isolates gave positive result while there are 6 isolates gave negative result by PCR. There were 4 isolates gave positive result by PCR for TEM gene while gave negative result by phenotypic test. without 10 μg of clavulanic acid (CAZ+CA).

**Table (2). Presence/absence of SHV, CTX-M and TEM genes in samples resistant/susceptible to the third generation Cephalosporins by PCR**

<table>
<thead>
<tr>
<th>Total samples (N= 133)</th>
<th>SHV</th>
<th>CTX_M</th>
<th>TEM</th>
<th>TEM, SHV</th>
<th>TEM, CTX-M</th>
<th>SHV, CTX-M</th>
<th>TEM, SHV, CTX-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESBL positive (N=46)</td>
<td>3</td>
<td>28</td>
<td>11</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ESBL negative (N=87)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Figure (4).** 2% agarose gel electrophoresis of *E.coli* isolated CTX-M genes by PCR. The lane one Marker: MW 100-300bp fragments. The lane two is control negative. The lane 3,4, and 5 they are isolates negative for CTX-M gene. The lane 6,7 and 8 with a band typical in size (550bp) which are positive for CTX-M gene.
Figure (4.7). 2% agarose gel electrophoresis of *E. coli* isolated TEM genes by PCR. The lane one Marker: MW 100-300bp fragments. The lane two is control negative. The lane 2, 4, 6, 7 and 8 they are isolates negative for TEM gene. The lane 3 and 5 with a band typical in size (971bp) which are positive for TEM gene.

Figure (4.8). 2% agarose gel electrophoresis of *E. coli* isolated SHV genes by PCR. The lane one Marker: MW 100-300bp fragments. The lane two is control negative. The lane 3, 5, 6, 7 and 8 they are isolates negative for SHV gene. The lane 4 with a band typical in size (850bp) which are positive for SHV gene.

3-DISCUSSIONS
In this study *E. coli* was the most common pathogen (57.3% of the Gram-negative isolates, 40% of all isolate). This goes with results that obtained in Sudan where *E. coli* was 77.7% of the Gram-negative isolates, 42.4% of all isolate (Hamdan et al., 2011). And near to that obtained in Tanzania where *E. coli* was 38% of the Gram-negative isolates and 25% of all isolate (Blomberg et al., 2005). A phenotypic confirmatory test is recommended by NCCLSs (now CLSI) is mandated to confirm the presence of ESBL by using ceftazidime and cefotaxime with and without clavulanic acid (Ghatole et al., 2004). In the present study
PCDDT (Phenotypic confirmatory disk diffusion test) detected 48/62 (77.4%) potential ESBL producers to be ESBL positive by this confirmatory test, with a sensitivity of 74.1% (46/62) for cefotaxime and it is combination and 78.4% (40/51) for ceftazidime and it is combination, as compared to screening tests. This result correlates well with Ravi et al., (2012), who find that the sensitivity cefotaxime 79.83 (198/248) and for ceftazidime 89.49 (196/219), as compared to sensitivity tests. Garrec and his colleagues (2011) achieved a sensitivity of 96% when testing at least Cefotaxime, Cefepime, and a third compound (Cefazidime, Cefpodoxime, or Aztreonam). While In a study from Latin America done by Winokur., et al. (2001) 55% of E. coli isolates gave positive result by PCT. Therefore, in order to increase the sensitivity to 100%, they proposed a two-step strategy using phenotypic methods. The lack of constant sensitivity of different phenotypic methods has also been emphasized by some other studies (Amaral et al., 2009). The 4 isolates that failed to show a clavulanate effect, probably represents other mechanisms of cephalosporin resistance that are not subject to clavulanate inhibition. These may include genes encoding OXA enzymes (Bush et al., 1995: Livermore., 1995: Naas., 1999) or AmpC-type enzymes (Bush et al.; Nordmann., 1998: Tzouvelekis, et al., 2000). The results of this study confirm the high prevalence of CTX-M gene, while the prevalence of other SHV genes was very low. The proportion of CTX-M strains among ESBL-producing E.coli isolates has dramatically increased from 38.2% to 87% (Moubareck, et al., 2005; Pitout, et al., 2005; Romero, et al., 2005). However in this study CTX-M genes occurred at high frequencies in E.coli with an account of 30/46 (65.2%), this result agree with Ben-Ami et al., who find 65% of ESBLs is CTX-M, (2009) Carolina, et al.,(2009), who find that the percentage of CTX-M 62%, and Kola, et al.,(2007) who find that the percentage of CTX-M gene was 70% among ESBLs. In contrast to the findings of above studies the prevalence of CTX-M type ESBL was less in a study in India by Sekar et al. (2009) who reported that 44.4% of E. coli. In this study TEM genes was a second isolates percentages among ESBLs 13/46 (28.2%), this result agree with Kiratisin et al.,(2008) in Thailand and disagree with Sekar et al. (2009) who find that TEM gene is the most predominant gene. SHV gene was the less gene in E.coli in this study 3/46 (6.5%) this in agree with many studies round the world, in Thailand by Kiratisin et al., 2008, (3.8%), in Iran by Dezful, et al., (2011) SHV was 9%. These results were different from other studies (Tasli and Bahar., 2005; Ben-Ami et al., (2009) which detected SHV-type ESBL in 74.3% of E.coli isolates.
CONCLUSION

This study confirms that E.coli is most UTI causative bacteria, the large dissemination and high percentages of the gene CTX-M among E. coli in Wad Alabass locality and that ESBLs have become a widespread serious problem and several aspects of them are worrying.

RECOMMENDATIONS

High prevalence of ESBL-positive E.coli was observed in our hospital setting. As the available treatment options are limited, antibiotic control policies together with the implementation of infection control measures and even isolation of infected patients remain of high importance. We must be conscious about administration and consumption of antibiotics in the treatment of infections especially UTI, in order to prevent the emergence of multi β-lactamases producing isolates and to reduce the chance of mutations in β-lactamases genes by reducing the antibiotics consumption. DNA sequencing and specific target genes primers are required to differentiate between ESBLs and ESBLs variant of TEM and SHV genes.

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