

## Prevalence of TEM, SHV and CTX-M genes in *Escherichia coli* and *Klebsiella* spp Urinary Isolates from Sudan with confirmed ESBL phenotype

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**Abstract:** The aim of the present study was to identify some of the genes, namely: CTX-M, SHV, and TEM, responsible for extended-spectrum beta-lactamase (ESBL) phenomenon among *Escherichia coli* and *Klebsiella* spp isolated from Sudanese patients infected with urinary tract infection (UTI). Two hundred and eighteen Gram negative urinary isolates were collected at different hospitals in Khartoum State. Identification of the isolates was done by using conventional biochemical methods, and microbact 2000 24E system from Oxoid. ESBLs were screened according to CLSI guidelines. ESBLs Positive strains were tested for the presence of ESBL encoding genes using PCR with specific primers for the detection of CTX-M, SHV and TEM genes. Out of 218 *Escherichia coli* and *Klebsiella* spp, ESBL was demonstrated in 130 (59.6%) of the isolates. The presences of CTX-M, SHV and TEM genes was confirmed in 68 (52.3%) of the isolates. The ESBL genes were detected in 19 *Klebsiella* spp and in 49 of *Escherichia coli* isolates. The communist frequent ESBL gene was CTX-M which was 48 and was observed in 35 and 13 of *Escherichia coli* and *Klebsiella* spp., respectively. The frequency of TEM gene was 38 and observed in 27 of *Escherichia coli*, and 11 of *Klebsiella* spp isolates. The frequency of SHV gene was 15 and observed in 3 and 12 of *Escherichia coli* and *Klebsiella* spp, respectively. It was concluded that all these genes were found to be carried by *K. pneumoniae* and *Escherichia coli* species. ESBL found to be higher in Sudan in comparison to other countries. Among urinary isolates the communist prevalence ESBL gene was CTX-M gene followed by TEM while the least one was SHV gene.

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### Introduction

Disease-causing microbes that have become resistant to antibiotic drug therapy are an increasing public health problem. UTIs are just a few of the diseases that have become hard to treat with antibiotics (Foxman, 2010). The situation in Sudan is of particular concern because self-medication and the use of antibiotics without medical guidance are largely facilitated by inadequate regulation of the distribution and sale of prescription drugs (Awad et al., 2007). Clinical microbiologists increasingly agree that multidrug resistant (MDR) Gram-negative bacteria pose the greatest risk to public health with faster increase in resistance (Cornaglia, 2009). The ESBLs are  $\beta$ -lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics and inhibited by  $\beta$ -lactamase inhibitors such as clavulanic acid (Giske et al., 2009). Resistance genes are often carried on bacterial plasmids, which are mobile elements of

DNA with the ability to readily spread through bacterial populations and between different bacterial species. Plasmid-encoded ESBL production, which confers resistance to most  $\beta$ -lactam antibiotics, was first identified in Germany in the 1980s (King et al., 2012). The class A beta-lactamases are most common group and large family consisting of TEM, SHV and CTX-M  $\beta$ -lactamases, in addition to other number of rare enzymes that often exhibit ESBL activity. The best way to define and identify the presence of a  $\beta$ -lactamase gene is by genetic methods PCR and sequencing which are standard methods for the determination of specific  $\beta$ -lactamase genes in bacterial isolates (Alfaresi and Elkoush, 2010).

### Materials and methods

In this study 218 Gram negative urinary isolates were collected from different hospitals in Khartoum State during the period of May to November 2011. Identification of bacterial isolates was done by using conventional biochemical methods and MICROBACT™ 24E system from Oxoid. After

identification, all Isolates were stored at  $-70^{\circ}\text{C}$  in Tryptic Soy broth with 20% glycerol till further tests. ESBLs were screened by detection of a reduced of zones of inhibition around third generation cephalosporins disks. Such strains were considered to be “suspicious for ESBL production” according to CLSI guidelines (CLSI, 2010). Double disk synergy test (DDST) was performed to confirm ESBL production as described by Jarlier et al. (1988). Isolates positive for ESBL production were screened by PCR using primers specific for the detection of *bla*TEM, *bla*CTX-M and *bla*SHV genes. DNA isolation was done, briefly, one ml of 24 hours Tryptic Soy Broth cultures, were transferred into 1.5ml sterile Eppendorff microfuge tubes and centrifuged at 10,000 rpm for 5 minutes. The pellets were dissolved in 600 $\mu\text{l}$  of lysis buffer (NaCl 1M, Tris-HCl 1M, EDTA 0.5M), 20 $\mu\text{l}$  SDS (25%), 3 $\mu\text{l}$  of proteinase K (20mg/ml) and incubated at  $60^{\circ}\text{C}$  for 1 hour. 620 $\mu\text{l}$  of phenol/chloroform/isoamylalcohol (25:24:1V/V) were added mixed and centrifuged at 10000 rpm for 10 minutes. The aqueous layer was transferred to another clean tube and 1ml of 95% of cold ethanol was added and allowed to stand for 1hour in at  $4^{\circ}\text{C}$ . DNA was then precipitated in each tube by centrifugation at maximum speed 10000 rpm for 10 minutes. DNA pellets were washed twice with 70% ethanol, dried and then re-suspended into 50 $\mu\text{l}$  of Tris-EDTA buffer and then stored at  $-20^{\circ}\text{C}$  until used (Shacheraghi et al., 2010). The primers used in this study were obtained from IDT Integrated DNA technologies (IDT, Belgium). PCR was carried in 50 $\mu\text{l}$  PCR reaction volumes containing 3 $\mu\text{l}$  of template DNA, 1 $\mu\text{l}$  (100 pmol) of each primer and a 25 $\mu\text{l}$  of Taq PCR Master Mix. Amplification of DNA was performed using Mastercycler Personal Thermal Cycler (Eppendorhoff, Germany). The PCR was carried out under the following conditions: initial

denaturation at  $95^{\circ}\text{C}$  for 5 minutes, followed by 30 cycles of denaturation at  $95^{\circ}\text{C}$  for 30s, primer annealing at  $55^{\circ}\text{C}$  for both *bla*TEM and *bla*SHV) or  $51^{\circ}\text{C}$  for *bla*CTX-M for 30s and primer extension at  $72^{\circ}\text{C}$  for 1 min. The time of extension step was increased to 10 min in the final cycle. TEM primers (TEMF ATGAGTATTCAACATTTCCGTG, TEMR TTACCAATGCTTAATCAGTGAG) amplified at 840-bp fragment, while SHV primers (SHVS1 ATTTGTCGCTTCTTTACTCGC, SHVS2 TTTATGGCGTTACCTTTGACC) amplified at 1051-bp fragment and CTX-M primers (CTX-MF TTTGCGATGTGCAGTACCAGTAA, CTX-MR CGATATCGTTGGTGGTGCCATA) amplified at 544-bp fragment (Sidjabat et al., 2010).

25 $\mu\text{l}$  of the PCR products were mixed 10  $\mu\text{l}$  of loading dye and analyzed by electrophoresis in 1% agarose gels (for 35 minutes at 90 V using 5 X TBE running buffer. 100 bp DNA ladder was included in each run, and DNA bands were viewed under UVP BioDoct It Imaging System after staining with ethidium bromide (2 g/ml).

### Results

In the present study, ESBL phenotype was demonstrated in more than half (59.6%) of the bacterial isolates. Frequency of different isolated species and their rates of ESBL phenotype are shown in (Table 1). High rate of ESBL was seen in *K. pneumoniae* 68.8 %, followed by *Escherichia coli* 65.0%, *P. mirabilis* 33.3%, *K. oxycota* 28.6%, and *P. aeruginosa* 10.0 %. PCR for TEM, CTX-M and SHV genes was positive in 68 (52.3%) of the tested isolates. 19 of these strains were *K. pneumoniae* while 49 were *Escherichia coli*. The frequency of TEM, CTX-M and SHV genes are fairly similar in *K. pneumoniae*, while in *Escherichia coli* CTX-M and TEM were the most frequently encountered genes (Table 2).

Table 1. Frequency of the different gram negative species included in the study and their ESBL production rates.

Species	Total Number	ESBL % (number)
<i>E. coli</i>	157	65.0 (102)
<i>K. pneumoniae</i>	32	68.8 (22)
<i>K. oxycota</i>	07	28.6 (02)
<i>P. mirabilis</i>	09	33.3 (03)
<i>P. aeruginosa</i>	10	10 (01)
<i>Enterobacter spp</i>	03	00.0 (0)
<b>Total</b>	<b>218</b>	<b>130</b>

Table 2. Distribution of TEM, SHV and CTX-M ESBL types among ESBL positive isolates.

Gene	<i>E. coli</i> (49)		<i>Klebsiella</i> (19)		Total (68)
	No	%	No	%	No
TEM	27	55.1	11	58.0	38
SHV	3	6.1	12	63.1	15
CTX-M	35	71.4	13	68.4	48

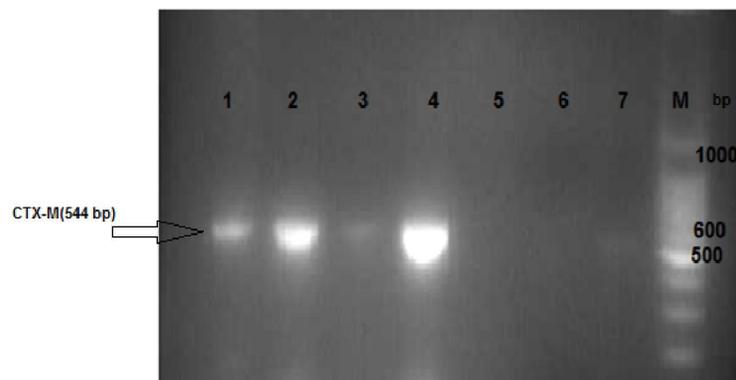


Figure 1. CTX gene after PCR on 1% Agarose gel electrophoresis. Lane M: 100-bp DNA ladder; lanes 2, 4, positive CTX-M gene at (544bp). Lanes 1: Control positive. Lane 7: control negative.

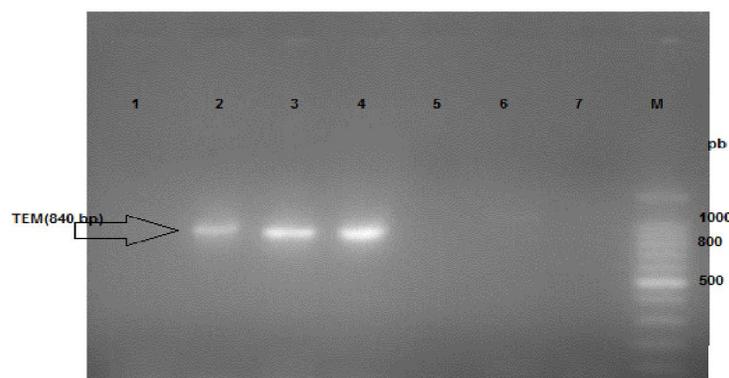


Figure 2. TEM gene after PCR on 1% Agarose gel electrophoresis. Lane: M, 100-bp DNA ladder; lanes 3 and 4 positive TEM gene at (840bp). Lanes 2: Control positive. Lane 7: control negative.



Figure 3. SHV gene after PCR on 1% Agarose gel electrophoresis. Lane: M, 100-bp DNA ladder; lanes 2, 4, and 6, SHV positive at (1051bp). Lanes 7: Control positive. Lane 5: control negative.

## Discussion

In the present study, ESBL phenotypes were found to be positive in 130 isolates (59.6%) while negative (non-ESB) phenotype was 88 (40.4%). A report from 10 European countries showed the prevalence of ESBL-producing *Escherichia coli* and *K. pneumoniae* ranged from at least 1.5% in Germany, and 39 to 47% in Russia, Poland and Turkey (Goosens, 2001). In 2010 Mekki and his colleagues reported ESBL production among Gram negative bacteria isolated from Sudan (Mekki et al., 2010). Dalela *et al.* from India (Dalela et al., 2012) and Özçakar ZB *et al.* from Turkey (Özçakar et al., 2011) reported rates of ESBL similar to the finding in this study.

Although multiplex PCR assay has been shown to have the advantage of rapidly screening large numbers of clinical isolates in addition to the fact that the isolated DNA would be suitable for further molecular epidemiological studies when required (Monstein et al., 2007), in this PCR with only one target was effectively used for the detection of different ESBL encoding genes.

In the present study, genotypic survey on 130 confirmed ESBL phenotype strains by PCR revealed 52.3% positive genotypes for at least one of studied genes. More positive strains were found among *K. pneumoniae* 68.8% compared to *Escherichia coli* 65.0% (Table 1). In a similar finding, Moosavian and Deiham found more positive strains in *Klebsiella* 79.5% (Moosavian and Deiham 2012).

In the present study 47.7% of positive phenotype ESBL strains lacked TEM, SHV and CTX-M genes, which can be explained by possible presence of other ESBL encoding genes in the studied bacterial population.

PCR results showed that the most common encountered ESBL gene in these urinary isolates was CTX-M. CTX-M gene was found in 71.4% of *Escherichia coli* strains and 86.4% of *Klebsiella* species. A study done by Ben-Ami et al. (2009) reported similar results. The CTX-M enzymes are known as an increasingly serious public health concern worldwide and have been noted to be the cause of outbreaks as reported elsewhere (Bonnet, 2004). The spread of CTX-M has also been described through prospective studies in industrialized countries such as Canada, France, and the United Kingdom (Ruppé et al., 2009).

The frequency of ESBLs TEM-type and SHV-type enzymes in this study were 28 and 15 respectively (Table 2). No information is currently available about detection of these genes in urinary isolates at Sudan. However, other studies showed prevalence of TEM and SHV genes in *K. pneumoniae* around 30.7 and 11.2% (Feizabadi et al., 2010), respectively and in *Escherichia coli* the rates were

46.4 and 11.2% (Shacheraghi et al., 2010), respectively. Some reports showed that most ESBLs were derivatives of TEM and SHV genes and they reached more than 90 TEM-type and more than 25 SHV-type  $\beta$ -lactamases (Dalela et al., 2012). In this study, the TEM genes were amplified from 55.1% of ESBL producing *Escherichia coli* and 58.0 % of ESBL producing *K pneumoniae* (Table 2). In the present study, the rate of TEM in *Escherichia coli* was lower than 87.5% (Tasli and Bahar, 2005) and close to 60% (Hosseini-Mazinani et al., 2007). Moreover, the rate of TEM-type  $\beta$ -lactamase produced by *K. pneumoniae* (58.0%) was higher than 31.1% that was found by Tasli and Bahar (2005) and less than 84.1% that was found by Al-Agamy et al. (2009). The results in this study showed prevalence of SHV-type ESBL among *Escherichia coli* strains was 6.1 % and among *Klebsiella* strains was 63.1% (Table 2). Bali et al. also found that SHV type ESBL was frequent in *K. pneumoniae* 53.3% isolates (Bali et al., 2010).

In conclusion, the ESBL producing isolates detected by PCR in 52.3% of the total isolates. PCR is a valuable tool for characterization of ESBLs in clinical and research settings. Determination of TEM and SHV genes by molecular techniques in ESBL producing bacteria may give useful data about their epidemiology and risk factors associated with these infections. Therefore, ESBL producing organisms should be promptly identified for appropriate antibiotic prescription and proper implementation of infection control measures (Rawat and Nair, 2010).

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