Molecular Characterization of CTX-M ESBLs among Pathogenic Enterobacteriaceae isolated from different regions in Sudan

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This was a cross sectional study conducted to detect and characterize CTX-M genes among extended spectrum β-lactamases (ESBLs) producing Enterobacteriaceae isolated from different regions in Sudan. A total of 305 of Enterobacteriaceae clinical isolates were collected from different regions in Sudan. ESBLs production was initially screened by cefotaxime, cefepime and Ceftazidime, then confirmed by disk combination method and PCR. DNA sequencing was done to differentiate between blaCTX-M genotypes. Escherichia coli was the most predominant isolate (58%), followed by Klebsiella pneumoniae (26.6%), Citrobacter freundii (3.6%), Enterobacter species (6.2%) and Proteus species (5.6%). ESBLs were detected by disk combination method in 128/305 (42%) of the tested isolates; Khartoum State 23/36 (64%), Gizera State 54/100 (54%), Sinnar State 49/92 (53%) and White Nile State 2/77 (2.6%). Three quarters of the ESBLs producers (96/128) were positive for blaCTX-M genes by PCR. The blaCTX-M-15 gene was the most predominant gene 18/23 (78.3%), followed by blaCTX-M-14 3/23(13%), blaCTX-M-27 1/23 (4.3%) and blaCTX-M-98 1/23 (4.3%). There was a transition mutation (substitution of A with G at position 25) in the blaCTX-M gene (ID: KP309815), that affected protein structure. In conclusion blaCTX-M-15 was the most commonly encountered gene and widely spread in different Sudanese regions.

Keywords: blaCTX-M; Beta-Lactamases Genes; Enterobacteriaceae; Sudan

INTRODUCTION

Antimicrobial resistance is becoming a chronic problem in the therapeutic intervention of infections. Production of ESBLs is a common mechanism of resistance among Gram negative bacilli. Production of ESBLs by Enterobacteriaceae has already had serious clinical implications. Several studies have confirmed an association between ESBL-producing Enterobacteriaceae and treatment failure, excess mortality, prolonged hospital
stay and increased treatment costs (Schwaber and Carmeli 2007; Pena et al., 1997). Nosocomial and community-associated infections such as urinary tract infections are frequently caused by Klebsiella pneumoniae and Escherichia coli; infections caused by these species are usually treated with Cephalosporins, so resistance rates to these antimicrobial agents have also been increasing (Murray et al., 2007).

Extended-spectrum β-lactamases (ESBLs) of the CTX-M types have been reported increasingly in gram-negative bacteria, mostly in Escherichia coli (Bonnet 2004). All CTX-M-types are ESBLs genes and do not have corresponding non-ESBL progenitors like TEM and SHV-type ESBLs. Based on their amino-acid sequence diversity, there are 172 variants of CTX-M genes identified until August 25, 2016, according to https://www.lahey.org/studies/.

CTX-M-15 is the most important gene among CTX-M types that has been associated with epidemic and mosaic plasmids, and also may play a role in conferring Carbapenem resistance in isolates with outer membrane impermeability (Livermore and Woodford 2006; Gröbner et al., 2009). CTX-M-15 efficiently can hydrolyze both Cefotaxime and Ceftazidime unlike other CTX-M enzymes (Karim et al., 2001). In Sudan, there is paucity of information on CTX-M types; hence, this study was conducted to investigate the prevalence and types of CTX-M types in different Sudanese geographical regions.

MATERIALS AND METHODS

Bacterial Isolates

Three hundred and five Enterobacteriaceae isolates (Escherichia coli = 177, K. pneumoniae = 81, Citrobacter freundii = 11, Enterobacter species = 19 and Proteus species = 17) were collected from different non duplicated clinical isolates of patients from different Sudanese regions including Khartoum State (Haj Alsa fi hospital), Sinnar State (Wad Alabass hospital), White Nile State (Aburugba village) and Gizera State (Wad Madani hospital) between December 2012 to November 2013. Chromogenic agar media (Liofilchem Co. Italy) and biochemical tests (according to CLSI guidelines) were used for isolation and identification of bacteria (CLSI 2011). The bacterial isolates were obtained from patient’s urine, vaginal swab, and wound swab. A written consent was obtained from every patient before collection of the samples.

ESBLs screening and confirmation

Antibiotic sensitivity and the ability of these isolates to produce ESBLs were determined according to Kirby-Bauer disc diffusion method (Wayne 2008). The following antibiotics disks were used; Cefazidime (CAZ: 30µg), Cefotaxime (CTX: 30µg), Cefepime (CPM: 30µg), Meropenem (MRP: 10µg) Amikacin (AK: 30µg), Ciprofloxacin (CIP: 5µg), Chloramphenicol (C: 30µg), and Nalidixic acid (NX 30µg) (Hi Media Lab Ltd., India). All isolates for which zone diameter ≤ 22 mm for Cefotaxime or ≤ 22 mm for Ceftazidime or ≤ 18 mm for Cefepime were considered to have a positive screening test for ESBLs, and then they were subjected to Clavulanate confirmatory testing. Phenotypic testing of ESBLs production was performed using the combination discs method according to the recommended method of the Clinical and Laboratory Standards Institute (CLSI) (Wayne 2006). We used Cefotaxime alone (CTX 30µg) and with Clavulanic acid (CTL 30µg+ 10 µg), Cefepime alone (FEP 30µg) and with Clavulanic acid (FEL 30µg+ 10 µg), and Ceftazidime alone (CAZ 30µg) and with Clavulanic acid (CAL 30µg+ 10 µg) (Liofilchem Co. Italy).

Polymerase Chain Reaction (PCR)

All positive isolates by confirmatory tests were subjected to molecular screening for β-lactamases genes using polymerase chain reaction for family specific blaCTXM-like. DNA extraction was done by the guanidine chloride method as described by (Alsadig et al., 2014). The PCR was carried out using primers; 5’-SCSATGTGCGAYACCAGT-3’ and CTX-MA-2 5’-CCGCRATATGRTTGGTGTG-3’, (Cao et al., 2002), (where S is G or C, Y is C or T, and R is A or G) (Metabion, Germany) for blaCTXM-like genes, in a total reaction volume of 25µl; 5µl Master mix (iNTRON Biotechnology, Seongnam, Korea), 0.6µl of forward primer, 0.6µl of reverse primer, 2µl DNA and 16.8µl deionized sterile water. The PCR mixture was then subjected to initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 seconds, primer annealed at 57°C for 45 seconds, followed by a step of elongation at 72°C for 60 seconds, the final elongation was at 72°C for 5 min. PCR products were analyzed by electrophoresis on a 2% agarose gel in TBE 1X that contain 2.5 µl of (20mg/ml) ethidium bromide at 100V for 40 min. Bands were visualized under U.V trans illuminator. A positive control obtained from previously sequenced CTX-M gene and a negative control contains DW, primers and PCR mixtures were used.

DNA sequencing

DNA sequencing was performed for 23 positive CTX-M genes (7 from Sinnar, 7 from Khartoum, 1 from White Nile and 8 from Gizera). DNA purification in addition to standard DNA sequencing was performed for both strands of blaCTXM genes (550Bp) by Macrogen Company (Seoul, Korea).
Table 1. Characteristics of the blaCTX-M-producing Enterobacteriaceae isolates

<table>
<thead>
<tr>
<th>Species</th>
<th>Antimicrobial resistance, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTX</td>
</tr>
<tr>
<td>E. coli (n= 177)</td>
<td>75 (42)</td>
</tr>
<tr>
<td>K. pneumoniae (n= 81)</td>
<td>34 (42)</td>
</tr>
<tr>
<td>Enterobacter species (n= 19)</td>
<td>7 (37)</td>
</tr>
<tr>
<td>Proteus species (n= 17)</td>
<td>9 (53)</td>
</tr>
<tr>
<td>Citrobacter species (n= 11)</td>
<td>3 (27)</td>
</tr>
<tr>
<td>Total (n=305)</td>
<td>128</td>
</tr>
</tbody>
</table>

CTX, Cefotaxime; CAZ, Ceftazidime; FEP, Cefepime; NX, Nalidixic acid; K, Amikacin; CIP, Ciprofloxacin; MRP, Meropenem; C, Chloramphenicol; n, number of isolates; (%), percentage.

* There was a significant association between species and resistant to Chloramphenicol.

We selected only 23 blaCTX-M positives gene for DNA sequencing due to the limitation of resources.

Sequence alignment

The nucleotide sequences of the blaCTX-M beta-lactamases genes were searched for sequence similarity using nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Atschul et al., 1997). Nucleotide sequences were translated into the amino acid sequence by GeneMarkS, (John et al., 2001), version 4.25 (http://exon.gatech.edu/genemark/genemarks.cgi). Protein sequence similarity was searched with BLASTp, highly similar sequences from NCBI were subjected to multiple sequence alignment and evolutionary analysis using BioEdit software (Hall 1999). Mutation analysis and prediction of tertiary protein structure were done online by Project HOPE software (http://www.cmbi.ru.nl/hope/report/2064?10) (16).

Statistical Analysis

We used the chi-square tests of the SPSS version 21.0, (IBM SPSS 2012), to check the statistical significance. The p-value that considered significant was less than 0.05.

RESULTS

A total of 305 clinical isolates of Enterobacteriaceae were identified; E. coli = 58% (177/305), K. pneumoniae = 26.6% (81/305), Citrobacter freundii = 3.6% (11/305), Enterobacter species = 6.2% (19/305) and Proteus species = 5.6% (17/305) (Table 1). The presence of ESBLs genes was confirmed through phenotypic confirmatory test where 128/305 (42%) isolates were positive. The most predominant ESBLs positive were E. coli 77/128 (60.2%); followed by K. pneumoniae 33/128 (25.7%), Enterobacter species 7/128 (5.5%), Citrobacter species 3/128 (2.3%) and Proteus species 8/128 (6.3%). Out of 128 (42%) positive ESBLs isolates; 63.8% (23/36) were from Khartoum State, 54% (54/100) from Gizera State, 549/92 (53%) from Sinnar State, and 2/77 (2.5%) from the White Nile state.

Identification of blaCTX-M genes in the clinical isolates

Out of 128 ESBLs producing isolate, 96/128 (75%) were positive for blaCTX-M genes (550bp) by PCR (Fig. 1)

DNA sequencing and multiple sequence alignment

DNA sequencing of amplified blaCTX-M (550 bp) genes (N = 23) revealed high occurrence of blaCTX-M-15 78.3% (N=18, AJ41341.1, AIQ77714.1), blaCTX-M-14 (13.1%) (N = 3, AAY58238.1), blaCTX-M-27 (4.3%) (N= 1, BAI68283.1) and blaCTX-M-98 (4.1%) (N= 1, WP_032495798.1) (Table 2). There was a silent mutation (substitution of G with A at position 24), and transition mutation (substitution of A to G at position 25) in isolate-13 (ID: KP309815) from White Nile State. This mutation resulted in a substitution of Lysine with Glutamic acid, as shown in Fig. 1.

Mutation analysis

Project HOPE online software revealed that there are some differences between wild and mutant residues; the
Figure 1. PCR amplification of CTX-M genes on 2% agarose gel electrophoresis. Lane 1 DNA ladder: MW 100-1500bp. Lane 2 is a positive control. Lane 3 a negative control. Lane 4, 5, 6, 7, 8, 9 and 10 showing typical bands size of 550bp corresponding to the molecular size of CTX-M genes.

Table 2. Phenotypic and genotypic detection of bla<sub>CTX-M</sub> genes from different Sudanese cities

<table>
<thead>
<tr>
<th>Isolate</th>
<th>State</th>
<th>Sample</th>
<th>Screening tests</th>
<th>Phenotypic tests</th>
<th>CTX-M genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Sinnar</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Sinnar</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
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<td>Sinnar</td>
<td>Urine</td>
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<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Sinnar</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
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</tr>
<tr>
<td>K. pneumoniae</td>
<td>Sinnar</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Khartoum</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Khartoum</td>
<td>Wound swab</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Khartoum</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Khartoum</td>
<td>Wound swab</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Khartoum</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
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<tr>
<td>E. coli</td>
<td>Khartoum</td>
<td>Wound swab</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>White Nile</td>
<td>Wound swab</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Gizaera</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 98</td>
</tr>
<tr>
<td>E. coli</td>
<td>Gizaera</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Gizaera</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
<tr>
<td>E. coli</td>
<td>Gizaera</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
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<tr>
<td>E. coli</td>
<td>Gizaera</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 27</td>
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<td>E. coli</td>
<td>Gizaera</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 14</td>
</tr>
<tr>
<td>E. coli</td>
<td>Gizaera</td>
<td>Urine</td>
<td>CTX + CAZ + FEP+</td>
<td>CTL + CAL + FEL+</td>
<td>CTX-M 15</td>
</tr>
</tbody>
</table>

Abbreviations: CAZ= Ceftazidime, CTX= Cefotaxime, FEP= Cefepime, CTL= Cefotaxime+ Clavulanic acid, CAL= Ceftazidime + Clavulanic acid and FEL= Cefepime + Clavulanic acid.
wild type residue forms a hydrogen bond with the Glutamic acid at position 20. The size difference between wild-type and mutant residue makes the new residue in an in correct position preventing the same hydrogen bond from binding at the same position of the original wild-type residue. The wild type residue forms a hydrogen bond with the Histidine at position 74 (Figure 2 and 3).

**DISCUSSION**

The production of ESBLs is one of the most important mechanisms of antimicrobial resistance. The recent increasing number of ESBLs producing *Enterobacteriaceae* is attributed to the emergence of CTX-M beta-lactamase (Livermore and Hawkey 2005). CTX-M ESBLs are the most prevalent ESBLs worldwide (Paterson and Bonomo 2005). In Sudan there were many studies conducted to investigate the prevalence of ESBLs genes (Mekki et al., 2010; Omar et al., 2013, Abdelmonem et al., 2005; Hamedelnil and Eltayeb 2012). In this study *E. coli* was the most predominant ESBLs producer presenting 58% of all isolates. This may be due to the fact that *E. coli* producing CTX-M beta-lactamases is present as common intestinal flora being harmless to the host disseminating in
In this study, the resistance to Cefotaxime (45.6%) was higher compared to Ceftazidime (39.3%) and Cefepime (13%). A high rate of resistance to Cefotaxime (67.8%) has also been reported by (Mobalayan et al., 2007), after studying 786 ESBL-producing E. coli strains isolated in Tabriz hospitals between 2006 to 2007. Moreover, there was a high rate of Ceftazidime resistance (39.3%) among blaCTX-M-producing isolates, this could be due to high production of bla<sub>CTX-M-15</sub> in these isolates. bla<sub>CTX-M-15</sub> was reported by (Poiriel et al., 2002) to have some hydrolytic activity against Ceftazidime, this may explain the high Ceftazidime resistance rate observed in this study. Furthermore, Cephalosporins resistance rate and production of bla<sub>CTX-M</sub> genes in this study was higher in urban cities than rural areas. In Khartoum (Capital of Sudan) 23/36 (64%) of the isolates were resistant to Cephalosporins, followed by 54/100 (54%) in Madani (Gizera State) the second city in Sudan, and 49/92 (53%) in Wad Alabass Locality (Sinnar State), while lower resistance rate was observed in rural areas such as Abu Rugba village 2/77 (2.6 %). These findings could be due to the fact that cities are more crowded than rural areas that facilitate ESBLs spread, in addition to the increase in antibiotics consumption in cities where the antibiotics are available and affordable in contrast to rural areas where these antibiotics are either unavailable or very expensive, and this is clearly observed in the results of Abu Rugba village where they were neither hospitals nor pharmacies. In Sudan, unfortunately, the Cephalosporins and other antibiotics are over the counter medication which explains the overuse of the antibiotics (Hamedenil and Altayb 2012).

bla<sub>CTX-M</sub> genes were detected in 96/128 (75%) of the ESBLs producers in this study, these findings are similar to those reported in Argentinean public hospitals whereCTX-M accounted for roughly 70% of all ESBLs (Quinteros et al., 2003). The high prevalence rate of ESBLs is largely explained by the uncontrolled use of antibiotics and the absence of active infection control programs with good antibiotic policies. The ESBLs producers which were negative for bla<sub>CTX-M</sub> genes 32/128(25%) in this study may harbor other ESBLs genes which confirms the need for further research in this field.

bla<sub>CTX-M-15</sub> and bla<sub>CTX-M-14</sub> are by far the most important ones, virtually invading all human and animal compartments as well as the environment all over the world (Cantón 2008; Hawkey and Jones 2009). This study highlighted the alarming explosive spread of bla<sub>CTX-M-15</sub>-producing Enterobacteriaceae in different Sudanese regions; even in a remote village e.g., (Abu Rugba). bla<sub>CTX-M-15</sub> was the most predominant one that representing 18/23 (78.3%) of all bla<sub>CTX-M-like</sub> types. This could be probably one reason for the growing problem of antibiotic resistance.

The high prevalence rate of ESBLs is largely explained by the uncontrolled use of antibiotics and the absence of active infection control programs with good antibiotic policies. The ESBLs producers which were negative for bla<sub>CTX-M</sub> genes 32/128(25%) in this study may harbor other ESBLs genes which confirms the need for further research in this field.

In conclusion, this study showed high prevalence of bla<sub>CTX-M-15</sub> producing bacteria in different Sudanese regions. These findings necessitate the need for epidemiological monitoring and prudent use of antimicrobial agents to limit the spread of bla<sub>CTX-M-15</sub>-producing isolates. These results present an alarming situation in Sudan which indicates the needs for further research in the field accompanying prompt control measures to prevent infections with these resistant isolates.

**Ethics statement**

This study was specially approved by Biotechnology park-African City of Technology, Sudan (http://www.act.sd), since the samples from diagnostic centers, not directly from a human.

**Disclosure**

All authors have approved the final article and declared no conflicts of interest.

**Availability of data and material**

All sequences in this study were submitted to the NCBI database (http://www.ncbi.nlm.nih.gov/) and their accession as follows; KP317181, KP317185, KP317182, KP317183, KP317186, KP317198, KP317184, KP317187, KP317188, KP317189, KP317199, KP317190, KP309815, KP317201, KP317191, KP317192, KP317193, KP317202, KP317200, KP317194, KP317195, KP317196 and KP317197.

**Author’s contribution**

HNA carried out the molecular genetic studies, doing the sequence alignment. MAS and AIH drafted the manuscript. MMM conceived of the experiments design and PCR. NMA participated in the sequence alignment and supervised this research. NMA participated in the design and coordination. All authors read and approved the final manuscript.

**Competing interests**

None declared.
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