Original Research Article

# CTX-M B-LACTAMASE–PRODUCING ESCHERICHIA COLI IN SUDAN TERTIARY HOSPITALS: DETECTION GENOTYPES VARIANTS AND BIOINFORMATICS ANALYSIS

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## Abstract

Background: Extended spectrum β-lactamase (ESBL)-producing *Escherichia coli* (ESBL-EC) constitute an emerging public-health concern. Consider that ESBL genes type CTX-M types has been increased significantly in most parts of the world. Few data are available on the CTX-M variants circulating in Sudan. Objective: This study used polymerase chain reaction (PCR) and bioinformatics tools to identify blaCTX-M and its variants for Extended-spectrum β-lactamase (ESBLs) producing clinical isolates of Escherichia coli (E. coli) obtained from hospitals in Khartoum-Sudan. Methods: A total of 216 non-repetitive isolates were selected during 2007-2018. The phenotypic identification of ESBL production was confirmed according to CLSI guidelines. CTX-M genotype was analyzed by uniplex PCRs reactions subsequently sequences performed later sequences were analyzed using bioinformatics tools with nBLAST program, multiple alignments to determine CTX-M genotype variants . Nucleotide sequences were submitted to Gen Bank and accession numbers were obtained. Result: ESBL phenotype among 212 confirmed E.coli isolates was (34.9% of 212,n=74). (62.2% of 74, n=46) strains carried bla CTX-M genes, CTX-M genotype variants identified in this study as followed: blaCTX-M-15 gene was the most prevalent one (78.6%) followed by blaCTX 90 (14.3%) and CTX-M55 (7.1%),

Conclusion: This study reveled high ESBL occurrence among *E.coli* isolates, with CTX-M 15 the predominant variants and highlights the incidence of CTX-M-55 for the first time from Sudan.

Keywords: E. coli, PCR, ESBLs, CTX-M, Sudan, bioinformatics

**Introduction:**

β-lactam group are the most widely used class of antibiotics globally, approximately 50% of all prescribed antimicrobial belong to this group[1] production β-Lactamases are the most frequent mechanism of resistance to β-lactam antibiotics in Enterobacteriaceae, various β-lactamases have been reported, regarding *E. coli* the most common mechanisms of resistance is production of extended-spectrum beta-lactamases (ESBL) [2]. ESBLs are class A plasmid mediated enzymes that hydrolyze oxyiminocephalosporin beside all penicillins and monobactam antibiotics but are inhibited by clavulanic acid in vitro are enzymes capable of conferring bacteria resistance to penicillins, 1st, 2nd, 3rd generation cephalosporins, and monobactams, the majority of clinically isolated ESBLs are TEM, SHV or CTX-M types [3] Extended spectrum producing *Enterobacteriaceae* (ESBL-E) are pervasive worldwide, resulting in increased morbidity, mortality and healthcare costs, E. coli producing CTX-M type ESBLs are the most common clinically encountered [4, 5]. Genes encoding ESBLs are frequently found on the same plasmid as genes encoding resistance for other classes of antibiotics, As a result, ESBL producing E. coli is frequently multidrug resistant (MDR), posing particular difficulties in the treatment of infections, especially in critically ill patients CTX-M-type ESBLs the most frequent ESBL type worldwide ,as its predominant cefotaximase activity and the location of its isolation (Munich) the enzyme was named,they are classified into five phylogenetic clusters (groups 1, 2, 8, 9, and 25), and alleles are numbered sequentially as they are discovered, CTX-M-15 and -14 are the most common CTX-M variants globally [6, 7].

In African countries like Sudan, there is generally a lack of comprehensive data with regards to ESBL-producing *E. coli* especially the variants of ESBL CTX-M genotype, few distributed studies reported (30.2 % of 232) ESBL phenotype among E.coli, without molecular characterization [8]. The only one published study that identified ESBL CTX-M genotype variants in Sudan reveled that ESBL

prevalence 42%, with CTX-M type represent (75% of 128) and the identified variants were blaCTX-M-15, was the most dominant (78.3% ,18/23), followed by blaCTX-M-14 (13%, 3/23), blaCTX-M-27, and blaCTX-M-98 with 4.3% (4.3%,1/23) for each[9].

This study focuses on detecting blaCTX M ESBL producing E.coli and identity their variants from clinical isolates obtained from four tertiary hospitals in Khartoum-state-Sudan to bring awareness to the decision maker about the intensity of the problem and to make interventions to curb the emergence and dissemination of CTX M ESBLs.

**Materials and Methods**

Study design

In this cross-sectional study that was conducted between March 2017 and Dec. 2017, a total of 216 clinical isolates (isolates were derived from different clinical samples of inpatients and outpateints) were collected from four hospitals in Khartoum-state –Sudan (Omdurman Teaching, Soba University, Royal care and Fedail ),. Two of the hospitals were a public and others private hospital, these four hospitals are tertiary care, and cover all Khartoum state localities and also they accept patients from allover Sudan, so they seem to be federal hospitals and can , so Reflect the situation of ESBL in almost all of Sudan. This study was approved by the ethical committee of. Confirm identity of these isolates was done based on culture characteristics on MacConkey agar and by standard biochemical reactions [10]. All required media and biochemical tests reagents were obtained from (HiMedia Labs, India).

Phenotypic detection of ESBL

Isolates were screened for ESBL production by using disc diffusion test on Muller Hinton agar according to CLSI guidelines[11] Isolates showing inhibition zone size of ≥22mm with ceftazidime (30μg), ≥25mm with ceftriaxone (30μg), ≥ 27mm with cefotaxime (30μg), were suspected for ESBL production. All screening test positive isolates were proceed for phenotypic confirmatory by

Inhibitor potentiated disc diffusion test (IPDD)[12].Briefly , The test inoculums (0.5 McFarland turbidity standard ) streaked onto two Muller—Hinton agar plates, one supplemented with 0.004 mg/L potassium clavulanate (United Tetra Group, Amman, Jordan ) and another without clavulanate. Ceftazidime (30g), cefotaxime (30g) and cefpodoxime (30g) discs were placed on both of these plates. The agar plates were then incubated at 37 ∘C overnight. The inhibition zones of the discs were compared between the plates with and without clavulanate. A difference of ≥10 mm in the zone diameter was confirmed as ESBL phenotype isolate .All required antibiotic discs were obtained from (HiMedia Labs, India).K. pneumoniae ATCC 700603 and E. coli ATCC 25922 were used as controls.

### Genotypic detection of bla$_{CTX-M}$ genes

Molecular tests conducted at National University Research Institute (NURI)-Khartoum-Sudan. All PCR reagents obtained from (iNtRON BIOTECHNOLOGY, Seongnam, Korea).DNA was extracted from pure colony of an overnight growth of confirmed ESBL phenotype using guanidine chloride method as described previously[13]. The concentration of extracted DNA was assessed by spectrophotometer [14]. Extracted DNA from ESBL-phenotype confirmed isolates were subjected for uniplex PCRs for the identification of CTX-M ESBL genes using universal primer using thermal cycler (Biometra-Germany), isolates that were positive for bla CTX-M universal gene , were further tested for blaCTX-M grouping using five uniplex PCRs for the identification of bla CTX-M groups (1,2,8,9 ,25), all PCR reactions were conducted in (50-µl) aliquots, using ready to use PCR Master Mix.

List of primers used for the amplification of blaCTX-M and its grouping with product size and annealing temperature , PCR reaction mixture for each DNA template and PCR program used in this study as shown on tables 1, 2 and 3 respectively [15-17].

**Table 1: List of primers used for the amplification of blaCTX-M and its grouping with product size and annealing temperature**

| Bla | Primer | Primer sequence | Amplicon size | Annealing temperature˚C |
|---|---|---|---|---|
| bla$_{CTX-M}$ universal | F | TTTGCGATGTGCAGTACCAGTAA | 590 | 59.2 |
| | R | CGATATCGTTGGTGGTGCCATA | | |
| blaCTX-M group 1 | F | GACGATGTCACTGGCTGAGC | 499 | 60.1 |
| | R | AGCCGCCGACGCTAATACA | | |
| blaCTX-M group 2 | F | GCGACCTGGTTAACTACAATCC | 351 | 59.8˚C |
| | R | CGGTAGTATTGCCCCTTAAGCC | | |
| blaCTX-M group 8 | F | CGCTTTGCCATGTGCAGCACC | 307 | No1: 64.4 No2: 57.4 |
| | R | GCTCAGTACGATCGAGCC | | |
| blaCTX-M group 9 | F | GCTGGAGAAAAGCAGCGGAG | 474 | 60.3 |
| | R | GTAAGCTGACGCAACGTCTG | | |
| blaCTX-M group 25 | F | GCACGATGACATTCGGG | 327 | No1: 61.2 No2: 54.2 |
| | R | AACCCACGATGTGGGTAGC | | |

**Table 2:  RCR reaction mixture for each DNA template**

| Component | Master mix | Forward primer | Reverse primer | DNA template | Sterile distilled Water | Total |
|---|---|---|---|---|---|---|
| volume( µl) | 10 | 2 | 2 | 4 | 32 | 50 |

**Table 3: PCR program used in this study**

| Step. No | Step | Temperature ºC | Time(min) | Cycles. No |
|---|---|---|---|---|
| I | Initial Denaturation | 95 | 5.0 | 1 |
| II | Denaturation | 94 | 1.0 | 35 |
| | Annealing | As shown in table 1 | 1.0 | |
| | Extension | 72 | 2.0 | |
| III | Final Extension | 72 | 10.0 | 1 |
| IV | Cooling  (Holding) | 10 ˚C | 60.0 | 1 |

Agarose Gel Electrophoresis

All PCR amplicons were verified by gel electrophoresis in 2% (w/v) agarose gel stained with 25 µg of ethidium bromide in tris-EDTA buffer for amplicons of the following sizes 590, 499, 351, 307, 474 and  327 for  $bla_{CTX-M}$ universal, $bla_{CTX-M}$ group1, $bla_{CTX-M}$ group 2, $bla_{CTX-M}$ group 8, $bla_{CTX-M}$ group 9 and $bla_{CTX-M}$ group 25 respectively ، which was performed at a voltage of 120V for 1 hour,, the bands on the gels were visualized by ultraviolet trans-illumination and photographed using gel documentation system. (Biometra-Germany). Further, 100 bp DNA ladder was included in each run. Positive and negative controls were amplified with each run.

Nucleotide sequencing of the amplicons:

The PCR products were sent for sequences at a commercial facility (Macrogen Company, Seoul, Korea), that done with same primers used in amplification.

Bioinformatics analysis

Determination of nucleotide sequence homologies:

In order identify of homologous sequences from sequence databases (Reference sequences),

the studied strains were marked by [SD number ], where , the reference sequences were marked by (accession numbers/country of origin, nucleotide sequences obtained were subjected to nucleotide basic local alignment search tool( blast n) analysis  using Nucleotide-nucleotide BLAST (blastn), that is bioinformatics tool available at

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome  [18].

Homology and evolutionary relation

In ordre to identify $bla_{CTX-M}$ genotype variants, Sequences of genes were analyzed for genetic relatedness corresponding each other and in compare to their references obtained from gene bank by Multiple sequence alignment and

evolutionary analysis through phylogenetic tree . these two bioinformatics analysis done through BioEdit software[19].The phylogenetic tree was constructed by neighbour-joining method with 1000 heuristic bootstrap replicates and substitution model as 'p distance[20].

Submission of sequences of the amplicons to the NCBI:

Nucleotide sequences were submitted to Gen Bank (www.ncbi.nlm.gov/genbank/) using BankIt submission tool (https://www.ncbi.nlm.nih.gov/WebSub/?form= history&tool=genbank ) and accession numbers were obtained.

Statistical analysis

All statistical analysess were performed using the Statistical Package for Social Sciences (version 22.0) software package for Windows (SPSS Inc., Chicago, IL). Data were expressed as percentages .Statistical analysis Categorical variables were compared using 0.05 was considered<Fisher's exact test. A p value of statistically significant [21].

The study was approved by Shendi University scientific research committee.

**Results**

Among the 216 E.coli isolates, 212 gave colonies of one type, while two gave colonies of two types, differing in morphology. Therefore, a total of 212 isolates were confirmed identity as E. coli.

ESBL prevalence

ESBL phenotype prevalence among E. coli isolates was (34.9% of 212, n=74) , (62.2% of 74, n=46) strains carried *bla CTX-M* genes ,CTX-M group 1 was the most dominant CTX-M group detected in 39 of 46 CTX-M positive isolates (85.8%), the rest (14.2%) CTX-M positive isolates carried CTX-M group 9 genes.

For sequences 12 selected from group1 and 2 from group 9 were proceed for bioinformatics analysis.

Representative agarose gel electrophoregram for *bla CTX-M* and their groups were shown on plates(1-3)
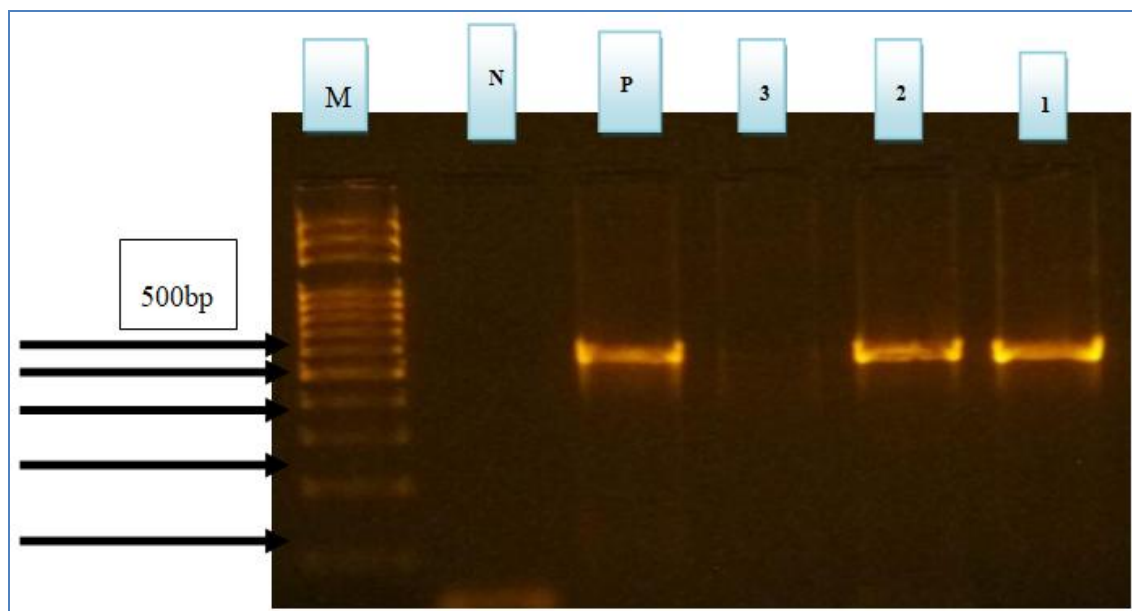


**Plate 1: 2% Agarose gel electrophoresis of PCR product for *bla* CTX-M (590 bp). Lane M:100 bp ladder, Lane P: bla$_{CTX-M}$ positive control, Lane 1–2: Positive isolates, Lane N: Negative control**
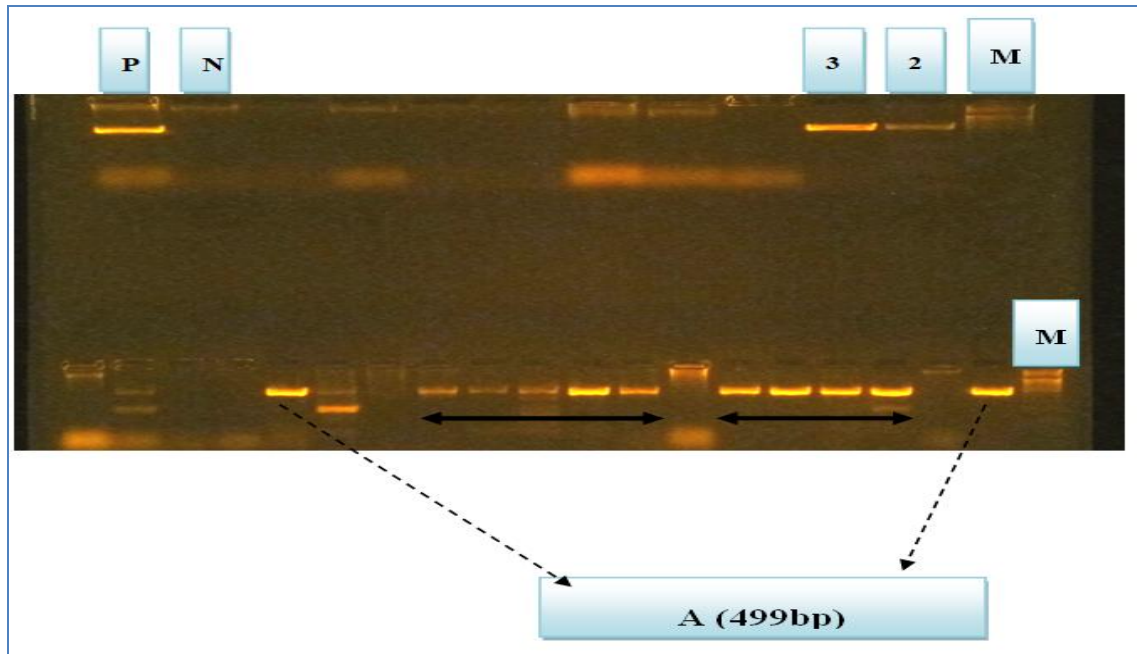
**Plate 2: Representative agarose gel electrophoregram of PCR product of ESBL gene CTX-M Group 1(band size 499bp)**

Lane M=100bp molecular weight marker (Ladder)
Upper bands:
Lane  2, 3, = PCR positive bla CTX-M group 1
Lane N =   Control negative
Lane P = control positive
Lower bands
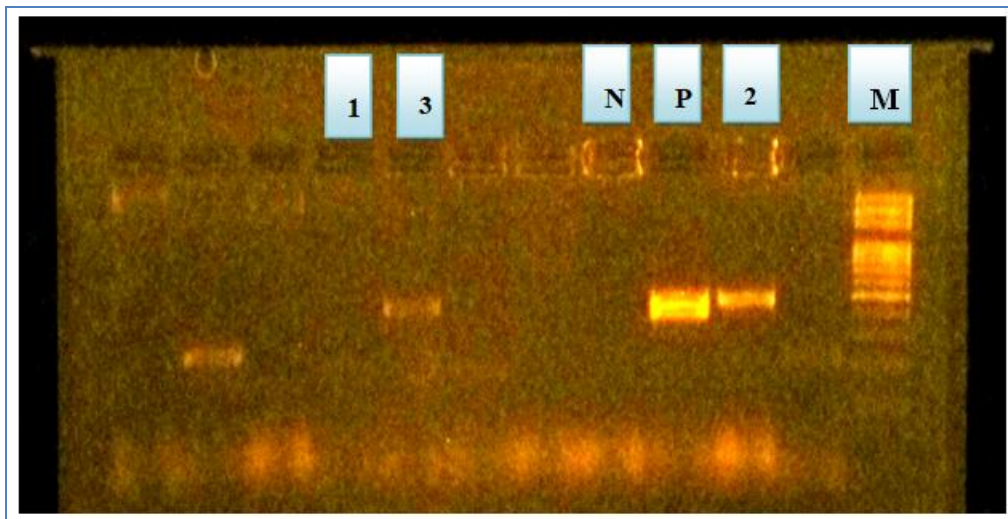11 bands (arrows) collected on (A) shape with size 499 bp  positive for bla CTX-M group1



**Plate 3 .Representative agarose gel electrophoregram of PCR product of ESBL gene CTX-M Group 9(band size 474bp)**

Lane M=100bp molecular weight marker (Ladder)
Lane 1= PCR negative bla CTX-M group 4
Lane 2, 3 = PCR positive bla CTX-M group 4
Lane P = Control positive
Lane N= Control negative

Bioinformatics analysis

Determination of nucleotide sequence homologies:

Blast n for (14 ) CTX-M sequences reveled  15 reference sequences , that all  fell into two major lineagesCTX-M-1 group (CTX-M- 15  and -55 ), CTX-M-9 group (CTX-M-9, -14, and -90),  CTX-M-15 allele was  the most prevalent  reference sequence .

Homology and evolutionary relation

Based on Multiple sequences alignment(MSA) and phylogenetic tree of all  CTX-M sequences(study and references)  figures(1- 2) reveled that  both CTX-M SD 8 and  CTX-M SD 13  were highly homology  to CTX-M-90 from china gb| MH898874.1, hence SD8 and SD13 identified as  CTX-M-90 allele.

Study strains CTX-M  SD6 , 9- 12 and 14-20 (sequence SD 15 omitted due to low quality sequence) were originated from  MK034764.1PakistanCTX-M-15 strain ,all identified as CTX-M 15 variants.

Noted that SD6 was close related to LC310946.1CTX-M-15,Iranian strain , while SD10and SD11 were close related to LC310944.1CTX-M-15 which is also Iranian strain.

 Only CTX-M.SD7 shown highly homology to CTX-M -55 from China, gb |MH898875.1, hence identified as CTX-M-55.

In summary among all SD CTX-M-sequences in this study (n=14) , proportion of  CTX-M variants in this study as followed : $bla_{CTX-M-15}$ genotype variant  was the most prevalent one (78.6% ) followed by $bla_{CTX}$ $_{90}$ (14.3%) and CTX-M55  (7.1%). (Figure 3)
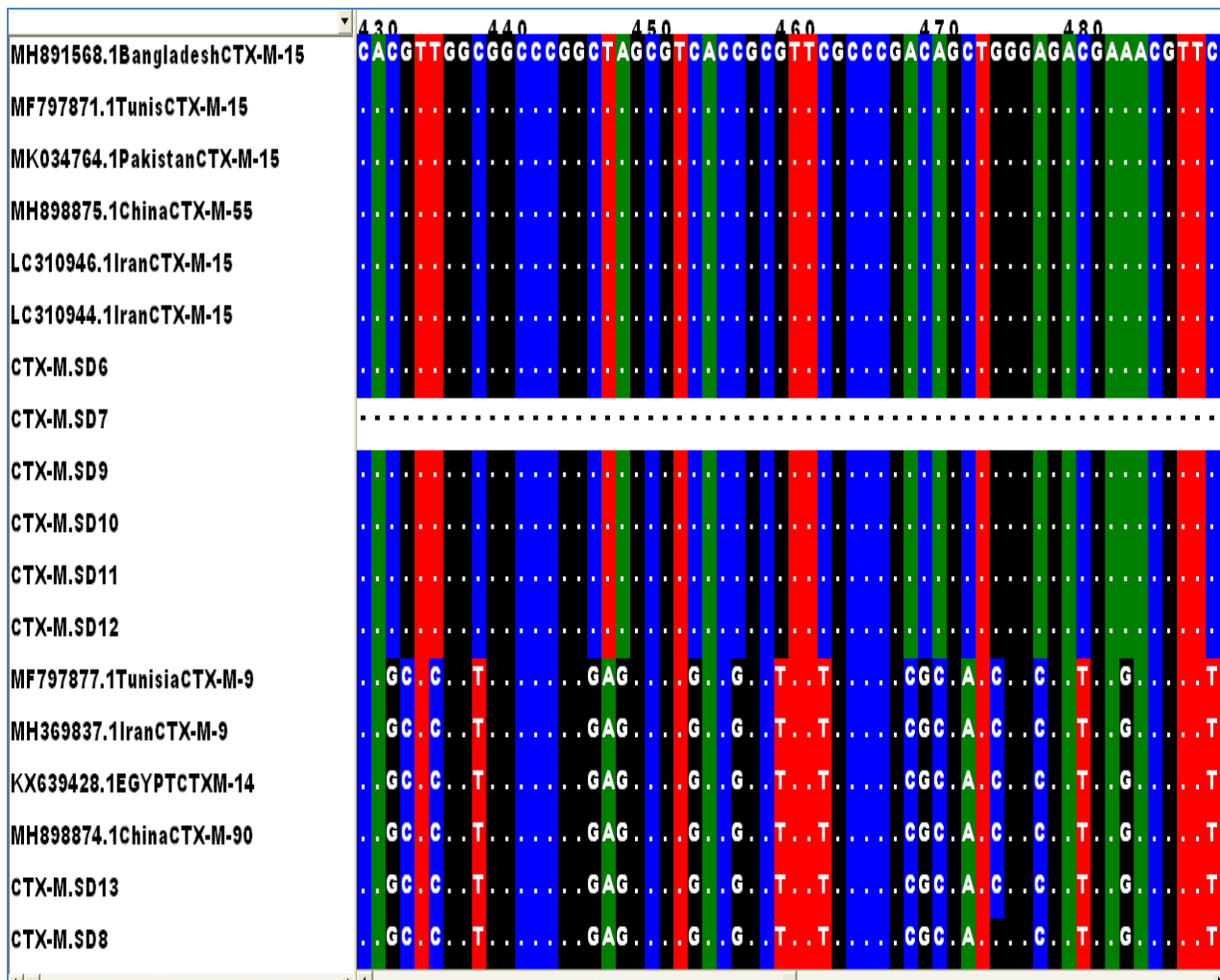


**Figure 1: Nucleotides multiple sequence alignment (MSA) of bla CTX-M sequences**
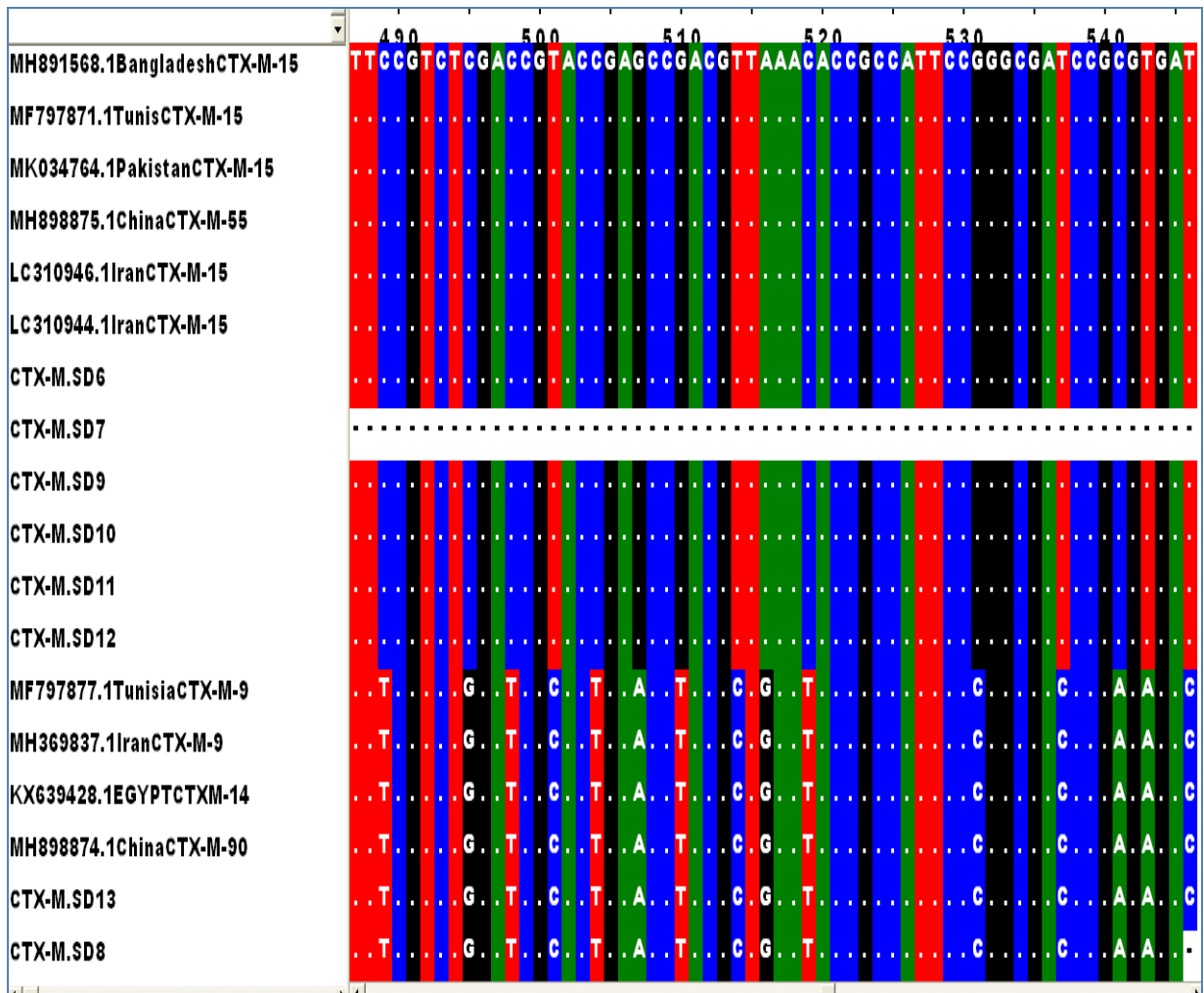
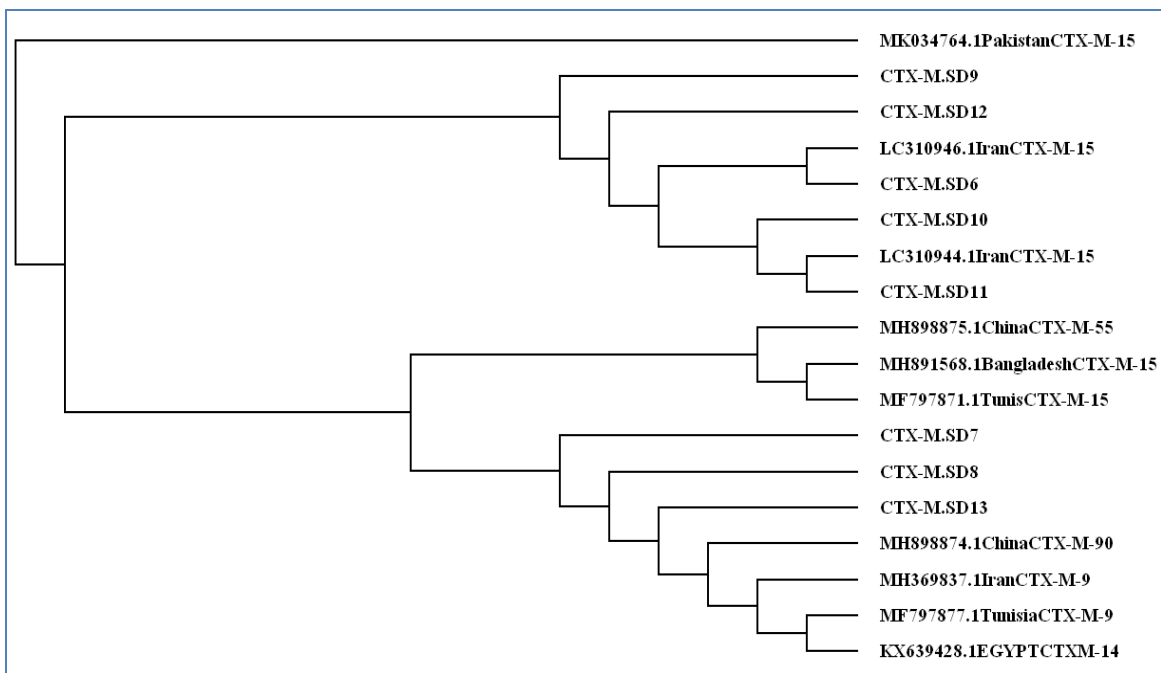Figure 2: Nucleotides multiple sequence alignment(MSA) of bla CTX-M sequences



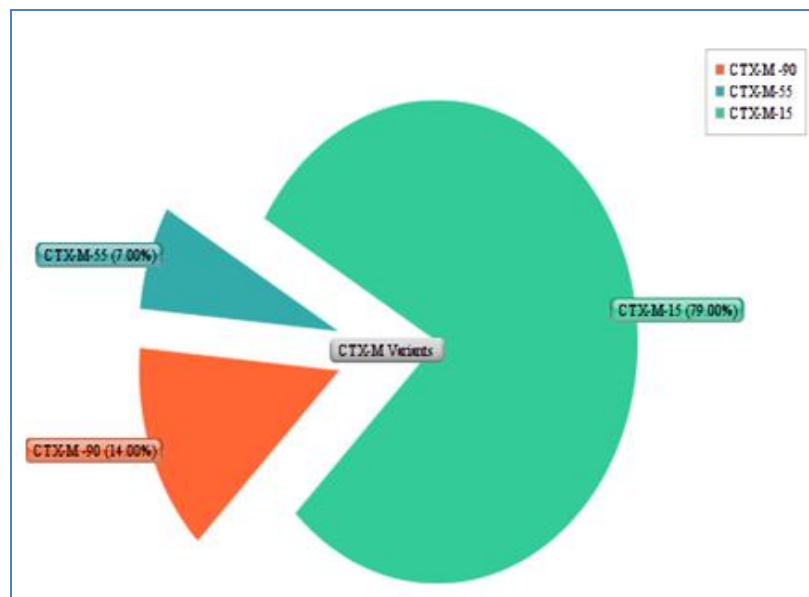Figure 3: Phylogenetic tree of bla CTX-M sequences

**Figure 4: Bla CTX-M genotype variants**

**GenBank accession numbers**

Will be released later

## Discussion

The predominant mechanism for acquired resistance to *β*-lactams in *Escherichia coli* is the synthesis of plasmid-borne extended-spectrum *β*-lactamases (ESBLs), ESBL-producing E. coli, particularly those producing CTX-M typeESBLs, are commonly associated with hospital- or community-related infection in humans[22]

This study presented the epidemiology of CTX-M *E. coli* from four hospitals in different localities of Khartoum state – Sudan. In this study, we observed that the majority of the ESBL-producing isolates (78%) were characterized as CTX-M-producers, supporting the recognition of CTX-M as the most prevalent type of ESBL in the world.

The prevalence of ESBL-producing *E. coli* was (34.9% of 212), which is consistent with the other study in Sudan[8].In contrast this study finding was much less than other one occurred in Sudan hospitals that report ESBL phenotype (65% of 157) and (60% of 128)[23, 24], which can be explained by different in study Isolates sources and techniques, and patient type (hospitalized or non hospitalized) .

In compare present study ESBL prevalence with region countries, In Kenya the median ESBL proportion was 45.8 %. Ethiopia 30.9 % , Uganda ( 61.7 %), (52% ) in Eygpt , Saudi Arabia (42.38%) ,Tanzania (45.2%) [25-27]. These differences may due to the difference in sensitivity and specificity between methods used in studies, some using only phenotypic methods, while others used both phenotypic and molecular-based method, others possibly; related to study participant and the variability in control use of antibiotics between these countries, where many antibiotics are still available over the counter in many regions of Africa.

This study reveled a high level of prevalence of CTX-M-type ESBLs among detected ESBL phenotype isolates ,in total, (62.7% of 74) of ESBL phenotype harbor CTX-M type, and the predominant group was CTX-M-1 group with variant CTX-M- 15 (78% of all CTX-M positive isolates). These findings were consistent with other study in Sudan that reported *bla CTX-*M proportion as (75 % of 128) and (71% of 49) [9, 28].

In this study, CTX-M-55 variant had been reported, what is believed to be the first time in

Sudan . This variant which is a derivative of CTX-M-15 and have amino-acid sequences that was identical of CTX-M-14 and CTX-M-18 and CTX-M-57 are was first identified in Thailand in 2007 from *E. coli* and Klebsiella pneumonia, recently reprted fro China , UK, Korea [29, 30]. Other variant which was repoted for first time from Sudan in this study was CTX-M -90 , this variant had been reported previously in Korea [31]. These variant which were identified for the first time, they may be detected before but missed in identification, in this study due to using of bioinformatics tool for sequences analysis so identification of any variant could be easier.

This shift from TEM/SHV to CTX-M, and in particular to CTX-M-15, has increased over time in most countries, and is dominant in most regions in accordance with many studies of ESBL-producing organisms allover the world Africa [7, 32, 33]

In conclusion, the high occurrence of ESBLs in Sudan cannot be ignored. There is an urgent need to adopt effective infection control measures. More such molecular studies with bioinformatics analysis must be conducted to determine ESBL epidemiology and their resistance trend which will help Decision makers to formulate antibiotic regimen policy.

This study demonstrates a remarkably high prevalence of *bla*CTX-M genes among ESBL-producing isolates. The high level of resistance to β-lactam and non-β-lactam antibiotics as well as the trend to a MDR profile associated with the *bla*CTX-M genes are alarming and emphasize the need for routine diagnostic antimicrobial susceptibility testing for appropriate choice of antimicrobial therapy.

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