Prevalence of $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{TEM}}$, and $\text{bla}_{\text{SHV}}$ Genes among Extended-spectrum $\beta$-lactamases-producing Clinical Isolates of Enterobacteriaceae in Different Regions of Sudan

Hisham N Altayb¹, Mohamed A M Siddig³, Nagwa M El Amin⁴, and Maowia M. Mukhtar⁵

¹Biochemistry Department, Faculty of Sciences, King Abdulaziz University, Jeddah, 21452, Saudi Arabia
³Botany department, Faculty of Science, University of Khartoum, Sudan
⁴Department of Microbiology, Faculty of Medicine, Qassim University, KSA
⁵Bioscience Research Institute, Ibn Sina University, P.O. Box 11463, Khartoum, Sudan

Abstract

Background: This study aimed to characterize $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{TEM}}$, and $\text{bla}_{\text{SHV}}$ genes among extended-spectrum beta-lactamases (ESBLs)-producing Enterobacteriaceae species in different regions of Sudan.

Methods: In this cross-sectional study, different clinical samples ($n = 985$) were collected randomly from symptomatic patients from four geographical regions of Sudan and cultured on chromogenic media. Following bacterial identification, phenotypic screening of ESBLs was done according to CLSI guidelines using cefotaxime (30 μg), ceftazidime (30 μg), and cefepime (30 μg) discs with and without clavulanic acid. The DNA was extracted by guanidine hydrochloride protocol, and then conventional PCR was used to detect $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{TEM}}$, and $\text{bla}_{\text{SHV}}$ genes. The presence of genes’ subtypes was characterized by DNA Sanger sequencing for selected samples.

Results: Enterobacteriaceae represented 31% (305/985) of all isolates, 42 (128/305) of which were ESBLs producer, confirmed by phenotypic confirmatory test (75% [96/128] of them were positive for $\text{bla}_{\text{CTX-M}}$ genes, 61% [78/128] for $\text{bla}_{\text{TEM}}$ genes, and 38% [48/128] for $\text{bla}_{\text{SHV}}$ genes). Fourteen isolates (11%) were negative for all genes. Forty-eight percent (63/75) of $\text{Escherichia coli}$ isolates were positive for $\text{bla}_{\text{CTX-M}}$, while in $\text{Klebsiella pneumoniae}$, the dominant gene was $\text{bla}_{\text{TEM}}$ (82%) and had a low amount of $\text{bla}_{\text{SHV}}$ (59%). There was a significant association ($P$-value = 0.001) for all except for chloramphenicol, $P = 0.014$, and amikacin, $P = 0.017$) between resistance to third-generation cephalosporins and ciprofloxacin, nalidixic acid, meropenem, chloramphenicol, and amikacin. Forty-two percent (40/96) of $\text{CTX-M}$-positive isolates were in Gizera State, 33% (32.96) in Sinnar, 24% (23.96) in Khartoum, and 1% (1/96) in White Nile.

Conclusion: We conclude that $\text{bla}_{\text{CTX-M}}$ genes are the most dominant genes in ESBLs-producing isolates and are more prevalent in big cities than in rural areas.

Keywords: phenotypic, $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{TEM}}$, and $\text{bla}_{\text{SHV}}$ ESBLs genes, Enterobacteriaceae, Sudan
1. Introduction

Extended-spectrum beta-lactamases (ESBLs) are the types of enzymes that cause resistance to most beta-lactam ring containing antibiotics [1]. Enterobacteriaceae sp. can resist a wide range of antibiotics, including cephalosporin and carbapenems, used as last-line antibiotics [2]. Infections caused by ESBLs-producing Enterobacteriaceae are increasingly being reported worldwide, causing high mortality rates, prolonged hospital stay, and rising medical costs [3]. *Escherichia coli* and *Klebsiella pneumoniae* species are considered among species associated with the high spread of ESBLs genes globally, especially the *bla*<sub>CTX-M</sub> genes, which have become more common in the last 20 years.

Recently, a dramatic increase has been reported in the frequency of *bla*<sub>CTX-M</sub> types β-lactamases-producing bacteria, which replaced the predominant types in the past, such as *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> [4]. *bla*<sub>CTX-M</sub> carrier, *E. coli*, can disseminate these genes in the community and hospitals from intestinal flora and cause infection [5].

Sudan is one of the many developing countries suffering from irrational use of antibiotics, where 63% of prescriptions contain antibiotics, and various forms of irrational cephalosporins usage are noticed [6, 7]. In Sudan, there is no regulation or system to govern antimicrobial use in humans or animals [8]. Resistance to cephalosporins and production of ESBLs genes in hospitals and environment have been reported in previous studies [9–11]. For the first time, this study aimed to detect *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>TEM</sub> genes and their subtypes among ESBLs-producing Enterobacteriaceae in different regions of Sudan.

2. Materials and Methods

A total of 975 clinical samples (*n* = 985; urine = 951, wound swab = 17, high vaginal swab = 12, pus = 3, sputum = 2) were collected from four different regions of Sudan – the Haj Alsafi Teaching Hospital in Khartoum (the capital of Sudan); Wad Madani Teaching Hospital in Wad Madani City (the second city in Sudan); Wad Alabass Hospital in Sennar State, Southern Sudan; and Abu Rugba village (remote village) in White Nile State. All samples were collected randomly from symptomatic patients (hospitalized or outpatients) during the study period and cultured on Chromogenic agar media (Liofilchem Co. Italy). Biochemical tests were applied for bacterial identification [12]. *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), and *K. pneumonia* (ATCC 700603) were used as quality-control strains.
2.1. Antimicrobial susceptibility testing

The antimicrobial susceptibility of the selected bacteria was done by the disc-diffusion technique [13]. The following antibiotic discs were used: amikacin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefepime (30 μg), chloramphenicol (30 μg), meropenem (10 μg), and nalidixic acid (30 μg) (Liofilchem Co. Italy).

2.2. Phenotypic detection of ESBLs

Phenotypic screening of ESBLs was done according to CLSI guidelines, the following discs were used: cefotaxime (30 μg), ceftazidime (30 μg), and cefepime (30 μg) discs with and without clavulanic acid. Phenotypically, ESBLs-positive isolates showed an increase of ≥5 mm in the zone around discs with clavulanic acid discs compared to the area around the disc without clavulanic acid [13].

2.3. Identification of extended-spectrum β-lactamase genes

All positive isolates with phenotypic confirmatory tests were subjected to molecular screening to detect β-lactamases genes using a conventional PCR machine. DNA isolation was done by the guanidine hydrochloride method, according to Sabeel et al. [14]. PCR was carried out using primer sequences presented in Table 1 (Metabion, Germany) for bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes [15, 16]. A reaction volume of 25 μl containing 5 μl Master Mix (iNtRON Biotechnology, Seongnam, Korea), 2 μl DNA, 0.6 μl of each primer, and 16.8 μl DW was used. The PCR steps were firstly subjected to 94°C for 5 min, then 30 cycles (94°C for 45 sec, 57°C for 45 sec, 72°C for 60 sec), and final elongation at 72°C for 5 min. PCR products were run at 2% agarose gel for bands detection by UV Transilluminator. Control positive (obtained from previously sequenced bla_{CTX-M}, bla_{TEM}, and bla_{SHV} genes) and control negative (containing DW, primers, and Master Mix) were used.

Sanger sequencing was achieved for both directions of DNA products by Macrogen Company (Seoul, Korea). DNA sequencing was performed for 25 bla_{CTX-M} (7 from Sinnar, 5 from Khartoum, 1 from White Nile, and 12 from Gizera), 3 bla_{SHV}, and 4 bla_{TEM} genes.
2.4. Statistical analysis

Data were analyzed by the statistical package for social science (SPSS) version 16, using the Chi-square test. A $P$-value $< 0.05$ was considered significant.

3. Results

While 68% (671/985) of the cultured samples showed a significant growth, 32% (314/985) showed no growth.

Of the 671 isolates, 305 (45.4%) were Enterobacteriaceae isolates ($E.\ coli = 177$, $K.\ pneumoniae = 81$, $C.\ freundii$ complex $= 11$, $Enterobacter$ species $= 19$, $P.\ mirabilis = 11$, $P.\ vulgaris = 6$); 133 (19.7%) were Enterococcus species; 177 (26.3%) were $S.\ aureus$; 19 (2.8%) were $Pseudomonas\ aeruginosa$; and 37 (5.5%) were yeast cells.

More than 45% (139/305) of all Enterobacteriaceae isolates were resistant to ceftaxime, 39.3% (120/305) to ceftazidime, and 13.4% (41/305) to cefepime. In addition, 92 (30.2%) isolates were resistant to ciprofloxacin, 79 (25.9%) to the nalidixic acid, 21 (6.9%) to meropenem, 99 (32.5%) to chloramphenicol, and 54 (17.7%) to amikacin. There was a statistically significant ($P$-value $< 0.05$) association between resistance to third-generation cephalosporins and resistance to other antibiotics used in this study ($P$-value $= 0.001$ for all except for chloramphenicol [$P = 0.014$] and amikacin [$P = 0.017$]) (Table 2).

3.1. Phenotypic confirmatory test of ESBLs

The occurrence of ESBLs genes was confirmed phenotypically in $>41\%$ (128/305) of the isolates; 54% (54/100) were in Gizera State (Madani), 63.8% (23/36) in Khartoum State (Haj Alsafi Hospital), 52.1% (48/92) in Sinnar State (Wad Alabas), and 1.3% (1/77) in White Nile State (Abu Rugba Village) (Table 3). There was a statistically significant ($P$-value $< 0.05$) association between ESBLs genes and the different regions of Sudan.

3.2. Genotyping of genes

Of the 128 isolates that showed ESBLs phenotype, 89% (114/128) were positive for ESBLs genes by PCR; 75% (96/128) were positive for $bla_{CTX-M}$ genes (Figure 1), 42% (40/96) of which were in Gizera, 33% (32.96) in Sinnar, 24% (23/96) in Khartoum, and 1% (1/96) in White Nile; 61% (78/128) were positive for $bla_{TEM}$ genes (Figure 2), and 38% (48/128) were positive for $bla_{SHV}$ genes (Figure 3; Table 4). Twenty-four isolates possessed only
\[ \text{bla}_{\text{CTX-M}} \text{ genes, seven only } \text{bla}_{\text{TEM}} \text{ genes, and one only } \text{bla}_{\text{SHV}} \text{ gene. Twenty-six isolates possessed the three genes together, seven harbored } \text{bla}_{\text{TEM}} \text{ and } \text{bla}_{\text{SHV}} \text{ genes, twenty-nine possessed both } \text{bla}_{\text{CTX-M}} \text{ and } \text{bla}_{\text{TEM}} \text{ genes, and ten harbored } \text{bla}_{\text{CTX-M}} \text{ and } \text{bla}_{\text{SHV}} \text{ genes. Moreover, 14 isolates (11%) gave negative results for the three genes.}

3.3. DNA sequencing

\[ \text{bla}_{\text{CTX-M-15}} \] represented 78.3% (18/23) of \[ \text{bla}_{\text{CTX-M}} \] genes, \[ \text{bla}_{\text{CTX-M-14}} \] 13.1% (3/23), \[ \text{bla}_{\text{CTX-M-27}} \] 4.3% (1/23), and \[ \text{bla}_{\text{CTX-M-98}} \] 4.3% (1/23). Out of the five \[ \text{bla}_{\text{TEM}} \] genes (971 bp), three isolates showed 100% identity with \[ \text{bla}_{\text{TEM-1}} \] (KM598665), while one isolate (isolate-29) showed 99% identity with \[ \text{bla}_{\text{TEM-1}} \] of \textit{E. coli} from China (AFI61435). DNA sequencing was also done for three \[ \text{bla}_{\text{SHV}} \] genes (797 bp); two isolates showed 100% identity (one with \[ \text{bla}_{\text{SHV-28}} \] [ACZ97629] and the other with \[ \text{bla}_{\text{SHV-1}} \] [ACZ97625.1]). The third isolate showed 98% identity with \[ \text{bla}_{\text{SHV-1}} \] (ACZ97624).

\begin{table}[ht]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Target} & \textbf{Primer name} & \textbf{Sequence} & \textbf{Product size (bp)} & \textbf{Annealing Temp.} \\
\hline
\text{CTX-M} & MA-1 F & SCSATGTCAGYACCAGTAA & 550 & 57°C \\
 & MA-2 R & CCGCRATATGRTTGGTG & & \\
\text{TEM} & C-F & TCGGGGAAATGTCGCAG & 971 & 55°C \\
 & D-R & TGCTAATCAGTGAGGCACC & & \\
\text{SHV} & OS-5-F & TTATCTCCTGTAGCCACC & 797 & 55°C \\
 & OS-6-R & GATTGCCGATTCGCTGG & & \\
\hline
\end{tabular}
\caption{Primer sequences used in the detection of genes.}
\end{table}

\begin{table}[ht]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Anti-microbial agent} & \textbf{ESBL producers} & \textbf{Non-ESBL producers} & \textbf{P-value} \\
 & \textbf{Resistant isolates} & \textbf{Resistant isolates} & \\
\hline
\text{Ciprofloxacin} & 66 (72%) & 26 (28.2%) & 0.001 \\
\text{Nalidixic acid} & 61 (77%) & 18 (22.7%) & 0.001 \\
\text{Meropenem} & 17 (81%) & 4 (19%) & 0.001 \\
\text{Chloramphenicol} & 70 (71%) & 29 (29.2%) & 0.014 \\
\text{Amikacin} & 43 (80%) & 11 (20%) & 0.017 \\
\hline
\end{tabular}
\caption{The association between antibiotic resistance and ESBLs-producing and non-ESBLs-producing bacteria.}
\end{table}
### Table 3: Phenotypic confirmatory test of ESBLs-producing isolates in different regions of Sudan.

| Region          | Confirmatory test | P-value |
|-----------------|-------------------|---------|
| Khartoum (n = 36) | CTX/CLA | 66 (72%) | 21 (58.3) | 0.001 |
|                 | CAZ/CLA | 19 (52.7%) |         |       |
|                 | CPM/CLA | 21 (58.3) |         |       |
| Gizera (n = 100) | CTX/CLA | 61 (77%) | 44 (44) | 0.001 |
|                 | CAZ/CLA | 45 (45%) |         |       |
|                 | CPM/CLA | 44 (44) |         |       |
| Sinnar (n = 92) | CTX/CLA | 17 (81%) | 44 (47.8) | 0.0001 |
|                 | CAZ/CLA | 43 (46.7%) |         |       |
|                 | CPM/CLA | 44 (47.8) |         |       |
| White Nile (n = 77) | CTX/CLA | 70 (71%) | 1 (1.3%) | 0.0001 |
|                 | CAZ/CLA | 1 (1.3%) |         |       |
|                 | CPM/CLA | 1 (1.3) |         |       |
| Total (n = 305) | CTX/CLA | 43 (80%) | 108 (35.4%) | 0.0001 |
|                 | CAZ/CLA | 108 (35.4%) |         |       |
|                 | CPM/CLA | 110 (36) |         |       |

CAZ: ceftazidime; CTX: cefotaxime; CPM: cefepime; CLA: clavulanic acid; ESBLs: extended spectrum beta-lactamases

### Table 4: Frequency of CTX-M TEM and SHV genes among ESBLs-producing Enterobacteriaceae isolates.

| Isolate                  | CTX-M | TEM | SHV |
|--------------------------|-------|-----|-----|
| *E. coli* (n = 75)       | 63 (84%) | 42 (56%) | 24 (32%) |
| *K. pneumoniae* (n = 34) | 21 (62%) | 28 (82%) | 20 (59%) |
| Enterobacter sp. (n = 7) | 5 (71%) | 4 (57%) | 1 (14%) |
| *P. mirabilis* (8)       | 4 (50%) | 3 (38%) | 2 (25%) |
| *P. vulgaris* (l)        | 1 (100%) | 0 (0%) | 1 (100%) |
| *C. freundii* complex (n = 3) | 2 (66%) | 1 (33%) | 0 (0%) |
| Total (n = 128)          | 96 (75%) | 78 (61%) | 48 (38%) |

**Figure 1:** PCR amplification of CTX-M genes. Lane 1 DNA marker (100–1500 bp), lane 2 positive control, lane 3 negative control, lanes 4–10 were positive for CTX-M genes (550 bp).

### 4. Discussion

Several studies exhibited that the prevalence of ESBL-producing bacteria is a serious problem of global public health, and their distribution can be varied according to geographic region, country, and studied institution [17, 18].
In this study, we report the increasing rates of ESBLs-producing *Enterobacteriaceae* compared to other previous study conducted in Khartoum State by Mekki *et al.* [19], we recorded ESBLs production among *Klebsiella* sp. and *E. coli* isolated as 53% in 2010. Moreover, Ahmed *et al.* [20] had recorded ESBLs production among *Enterobacteriaceae* sp. as 59.6% in 2013. This finding in the Sinnar State is higher than that previously reported in 2012 by Hamedelnil and Eltayeb [21], who reported that 36% of 133 isolates were ESBLs producers.

In the current study, we report that resistance to cefotaxime (45%) is higher than that to ceftazidime (39.3%) and cefepime (13.4%). We also observed a high ceftazidime-resistant rate (39.3%) within CTX-M-positive isolates, which may be because we report high frequencies of \(\text{bla}_{\text{CTX-M}}\) genes in our isolates. Previous reports indicated several types of \(\text{bla}_{\text{CTX-M}}\) genes exhibiting an increased enzymatic activity against ceftazidime.
A high resistance rate was observed in this study to ciprofloxacin, nalidixic acid, meropenem, chloramphenicol, and amikacin within ESBLs-producing isolates. There was statistically significant ($P$-value $< 0.05$) association between resistance to third-generation cephalosporins and these antibiotics ($P$-value $= 0.001, 0.001, 0.001, 0.014$, and $0.017$, respectively). The possible cause for this phenomenon may be that ESBLs are encoded on mobile plasmids, facilitating its transmission from one organism to another.

Furthermore, 89% (114/128) of ESBLs-positive isolates by phenotypic detection were also positive by PCR; $\text{bla}_{\text{CTX-M}}$ genes were positive in 75% (96/128), $\text{bla}_{\text{TEM}}$ genes in 61% (78/128), and $\text{bla}_{\text{SHV}}$ genes in 38% (48/128). This finding agrees with Hamedelnil and Eltayeb [21] and Omar et al. [20], who reported that $\text{bla}_{\text{CTX-M}}$ genes were the most dominant genes followed by $\text{bla}_{\text{TEM}}$ and $\text{bla}_{\text{SHV}}$ genes. High frequencies of $\text{bla}_{\text{SHV}}$ genes (67.4%) were reported in other studies; Feizabadi et al. [25] reported genes encoding the ESBLs, including $\text{bla}_{\text{SHV}}$ and $\text{bla}_{\text{CTX-M}}$ among 89 $K$. pneumoniae isolates by PCR.

There were 14 isolates (11%) that gave negative results by PCR but positive by phenotypic test for ESBLs; this may be due to other ESBLs genes that were not covered by our primers sets. Many studies have confirmed the presence of other ESBLs genes in Enterobacteriaceae like $\text{bla}_{\text{VEB-1}}, \text{bla}_{\text{OXA}},$ and $\text{bla}_{\text{PER}}$ or may be due to the presence of another mechanism of resistance [26, 27]. Furthermore, 19 ESBLs-positive isolates harbored only $\text{bla}_{\text{TEM}}$ and $\text{bla}_{\text{SHV}}$ genes; some of these genes were $\text{bla}_{\text{TEM-1}},$ and $\text{bla}_{\text{SHV-1}}$ and ESBLs phenomenon may arise from another mechanism of resistance.

In the present study, we observed that the cephalosporin-resistance rate and production of ESBLs genes were higher in urban cities compared to rural areas. In Khartoum, 64% (23/36) of the isolates were resistant to cephalosporin, 54% (54/100) in Madani (Gizera State), and 51% (48/94) in Wad Alabass locality (Sinnar State), while in rural areas such as Abu Rugba village, they were much lower (2.6 % [2/76]). This difference may be because cities are more crowded than rural areas, which facilitates ESBLs spread. Also, antibiotic consumption is higher in cities than in rural areas due to easy access to hospitals and pharmacies, and this is observed in the result of Abu Rugba village, where there is no pharmacy or hospital. Unfortunately, in Sudan, cephalosporins and other antibiotics are sold as over-the-counter medication, explaining the overuse of antibiotics [6, 7].
Limitations

Limited resources prevented us from sequencing all amplified genes, and we selected some samples from different regions to be used as control strains to give us a general idea about the common gene subtypes.

5. Conclusions

This study has shown a high prevalence of ESBLs-producing bacteria in different regions of Sudan, especially in big cities than in rural areas. bla\textsubscript{CTX-M} genes are the most dominant genes in ESBLs-producing isolates. This alarming situation of explosive spreading of ESBLs genes, especially bla\textsubscript{CTX-M}\textsuperscript{−} producing isolates, highlights the need for their epidemiological monitoring. Integrated and regular management of antibiotic consumption needs to be monitored in our society to limit their spread.

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Ethical considerations

The study protocol was approved by the Ethical Research Committee of Sudan University of Science and Technology (SUST/DSR/IEC/EA2/2014), Sudan. Because we collected the remaining medical samples with limited data, participant consent was waived.

Competing interests

None.

Availability of data and material

All sequencing data were uploaded on GenBank and assigned accession numbers found in the supplementary file.
Funding

None.

Authors’ contributions

HNA, MAM, NME, and MMM contributed to the study design; HNA did the experiments; HNA and NME contributed to data analysis; HNA, MAM, NME, and MMM contributed to manuscript writing; all approved the final version of this article.

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