Sudan Journal of Medical Sciences
Volume 16, Issue no. 1, DOI 10.18502/sjms.v16i1.8933
Production and Hosting by Knowledge E

Research Article

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

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Received 7 February 2021
Accepted 20 March 2021
Published 31 March 2021

Production and Hosting by Knowledge E

Editor-in-Chief:
Prof. Mohammad A. M. Ibnouf

Abstract

Background: This study aimed to characterize *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ 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 *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ 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 *bla*$_{CTX-M}$ genes, 61% [78/128] for *bla*$_{TEM}$ genes, and 38% [48/128] for *bla*$_{SHV}$ genes). Fourteen isolates (11%) were negative for all genes. Forty-eight percent (63/75) of *Escherichia coli* isolates were positive for *bla*$_{CTX-M}$, while in *Klebsiella pneumoniae,* the dominant gene was *bla*$_{TEM}$ (82%) and had a low amount of *bla*$_{SHV}$ (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 *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 *bla*$_{CTX-M}$ genes are the most dominant genes in ESBLs-producing isolates and are more prevalent in big cities than in rural areas.

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Keywords: phenotypic, *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ 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 $bla_{CTX\text{-}M}$ genes, which have become more common in the last 20 years.

Recently, a dramatic increase has been reported in the frequency of $bla_{CTX\text{-}M}$ types β-lactamases-producing bacteria, which replaced the predominant types in the past, such as bla_{TEM} and bla_{SHV} [4]. $bla_{CTX\text{-}M}$ 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 $bla_{CTX\text{-}M}$, bla_{SHV}, and bla_{TEM} 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 $bla_{CTX\text{-}M}$, 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 $bla_{CTX\text{-}M}$, 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 $bla_{CTX\text{-}M}$ (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 cefotaxime, 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 $bla_{CTX\text{-}M}$ 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

$bla_{CTX\text{-}M}$ genes, seven only bla_{TEM} genes, and one only bla_{SHV} gene. Twenty-six isolates possessed the three genes together, seven harbored bla_{TEM} and bla_{SHV} genes, twenty-nine possessed both $bla_{CTX\text{-}M}$ and bla_{TEM} genes, and ten harbored $bla_{CTX\text{-}M}$ and bla_{SHV} genes. Moreover, 14 isolates (11%) gave negative results for the three genes.

3.3. DNA sequencing

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

Table 1: Primer sequences used in the detection of genes.

Target	Primer name	Sequence	Product size (bp)	Annealing Temp.
CTX-M	MA-1 F	SCSATGTGCAGYACC AGTAA	550	57°C
	MA-2 R	CCGCRATATGRT TGGTGGTG		
TEM	C-F D-R	TCGGGGAAATGTGCGCG TGCTTAATCAGTGAGGCA CC	971	55°C
SHV	OS-5-F OS-6-R	TTATCTCCCTGTTAGCCACC GATTTGCTGATTTCGCTCGG	797	55°C

Table 2: The association between antibiotic resistance and ESBLs-producing and non-ESBLs-producing bacteria.

Anti-microbial agent	ESBL producers Resistant isolates	Non-ESBL producers Resistant isolates	*P*-value
Ciprofloxacin	66 (72%)	26 (28.2%)	0.001
Nalidixic acid	61 (77%)	18 (22.7%)	0.001
Meropenem	17 (81%)	4 (19%)	0.001
Chloramphenicol	70 (71%)	29 (29.2%)	0.014
Amikacin	43 (80%)	11 (20%)	0.017

Table 3: Phenotypic confirmatory test of ESBLs-producing isolates in different regions of Sudan.

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

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 (1)	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%)

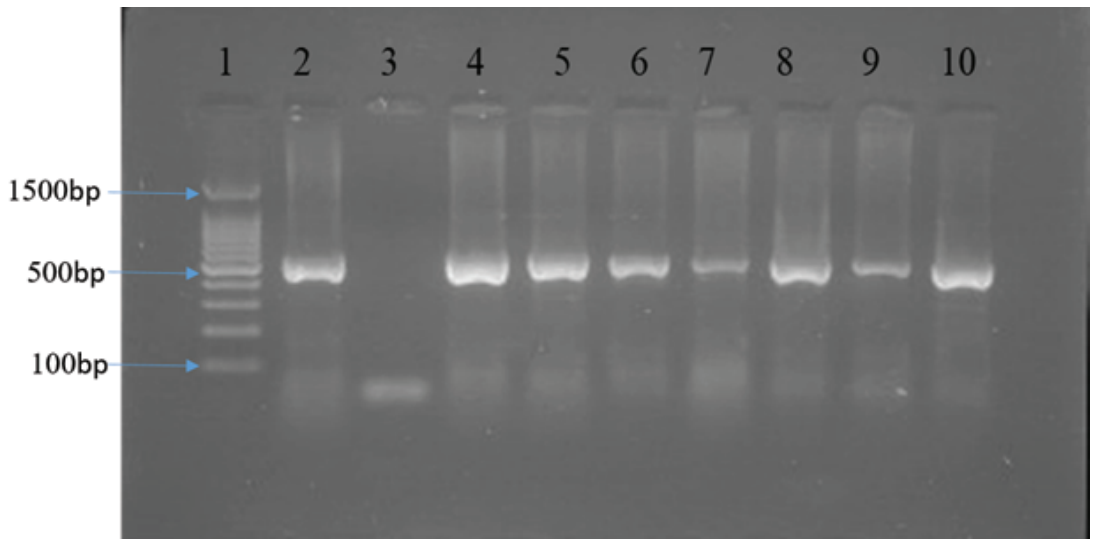

Figure 1: PCR amplification of *CTX-M* genes. Lane 1 DNA marker (100–1500 bp), lane 2 positive control, lane 3 negative control, lanes 4–10 were positive for *CTX-M* genes (550 bp).

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].

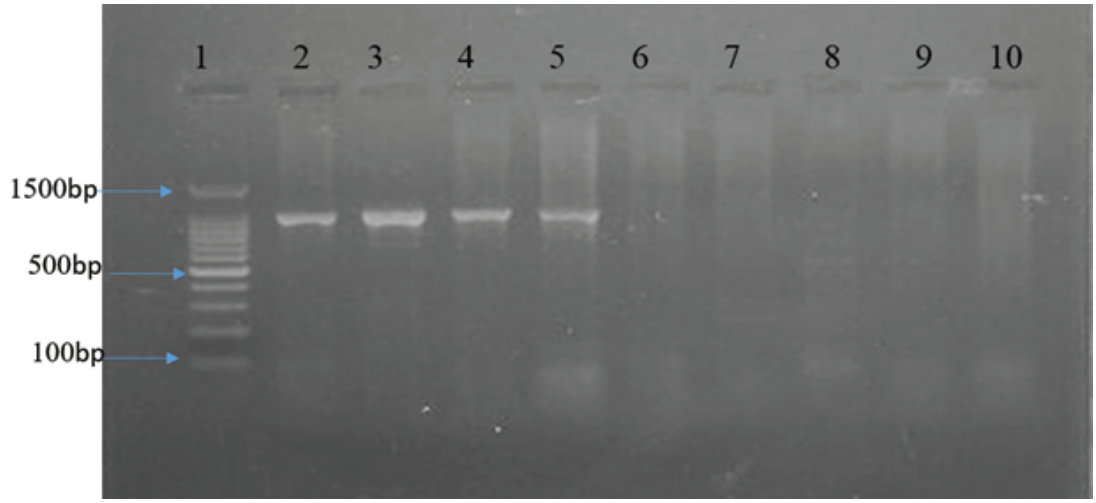

Figure 2: Amplification of *TEM* genes. Lane 1 DNA marker (100–1500bp), lane 2 positive control, lanes 3–5 were positive for *TEM* (971 bp), lanes 6–10 are negative samples.

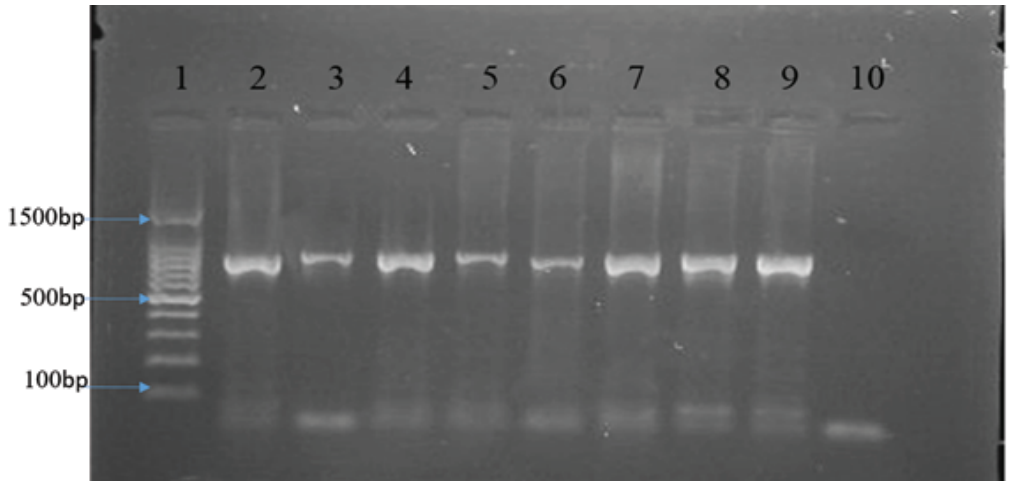

Figure 3: PCR amplification of *SHV* genes separated. Lane 1 DNA marker: MW 100–1500 bp, lane 2 positive control, lanes 3–9 were positive for *SHV* genes (797 bp), lane 10 is negative control.

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 $bla_{CTX\text{-}M}$ genes in our isolates. Previous reports indicated several types of $bla_{CTX\text{-}M}$ genes exhibiting an increased enzymatic activity against ceftazidime

[22, 23]. 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 [24].

Furthermore, 89% (114/128) of ESBLs-positive isolates by phenotypic detection were also positive by PCR; bla_{CTX-M} genes were positive in 75% (96/128), bla_{TEM} genes in 61% (78/128), and bla_{SHV} genes in 38% (48/128). This finding agrees with Hamedelnil and Eltayeb [21] and Omar *et al.* [20], who reported that bla_{CTX-M} genes were the most dominant genes followed by bla_{TEM} and bla_{SHV} genes. High frequencies of bla_{SHV} genes (67.4%) were reported in other studies; Feizabadi *et al.* [25] reported genes encoding the ESBLs, including bla_{SHV} and bla_{CTX-M} among 89 *K. pneumonia* 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 bla_{VEB-1}, bla_{OXA}, and bla_{PER} or may be due to the presence of another mechanism of resistance [26, 27]. Furthermore, 19 ESBLs-positive isolates harbored only bla_{TEM} and bla_{SHV} genes; some of these genes were bla_{TEM-1}, and bla_{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. **$bla_{CTX\text{-}M}$** genes are the most dominant genes in ESBLs-producing isolates. This alarming situation of explosive spreading of ESBLs genes, especially blaCTX$_{_M}$-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.

Acknowledgments

The authors are thankful to Hassuna Eltayeb from Abu Dhabi Media Co. for the language editing of the manuscript. They also acknowledge Dr. Hiba Salah-Eldin and Dr. Saher M Bakhiet for their support.

Ethical considerations

The study protocol was approved by the Ethical Research Committee of Sudan University of Science and Technology (SUST/DSR/1EC/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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