RESEARCH ARTICLE

Characterisation of Extended-Spectrum β-Lactamases among Multidrug Resistant Enterobacteriaceae from Sudan

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Abstract

The aim of the present study was to characterize extended-spectrum β-lactamase (ESBLs) genes in multidrug resistant enterobacterial pathogens as well as commensal isolates from the Sudan during the period 2003 to 2007. ESBL production was determined phenotypically by the combined disc method, and was characterized genotypically by the detection of *bla* genes by PCR and nucleotide sequencing. Transferability was examined by conjugation with nalidixic-acid resistant *E. coli* K12. The results showed that a total of 106 of the 113 (94%) isolates including *E. coli*, *Klebsiella pneumoniae*, *proteus* spp., *Enterobacter cloacae*, *Providencia* spp. and *Morganella morganii*, were positive for *bla* genes including the prototype *bla*TEM. Eleven isolates (28%) of the 113 were ESBL producers encoding *bla*SHV genes (SHV5, SHV5a, SHV12, SHV26, SHV28 and SHV38), 90 isolates (80%) were CTX-M positive. All, but only one (CTX-M9) were CTX-M15. Only 3(2.7%) of the isolates were Amp-C producers (CMY-4 and DHA-1). Plasmid transfer of the multiple resistance patterns was achieved among all the isolates. These findings demonstrated that ESBLs were highly produced by multi-resistant enterobacterial isolates from the Sudan; among both clinical pathogens as well as stool commensals. This is the first report of ESBLs genes characterization from the Sudan.

Keywords: Multidrug, *bla* genes, enterobacteria, ESBLs, pathogens, commensals, resistance.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) are β-lactamase enzymes capable of conferring bacterial resistance to the broad-spectrum oxyiminocephalosporins. They hydrolyse extended-spectrum cephalosporins, as well as aztreonam. They are inhibited by β-lactamase inhibitors such as clavulanic acid[1,2]. ESBLs are not active against cephamycins and most strains expressing ESBLs are susceptible to cefoxitin and cefotetan. However ESBL producing strains can become resistant to cephamycins due to acquisition of plasmid-mediated Amp-C β-lactamases or to the loss of outer membrane porin proteins[3]. Carbepenems are less susceptible to hydrolysis by ESBLs than any other β-lactam antimicrobials, although resistance has been reported among mutants with porin loss[3].

ESBLs represent a major health problem worldwide[4,5]. Over 60% of bacterial nosocomial isolates mostly *E. coli* and *Klebsiella spp* are claimed to be capable of beta-lactamase production[6,7]. ESBL-related infections are on the rise and has been observed throughout the globe on both hospital and community settings[8,9,10,11]. Community-acquired infections or colonization with ESBL-producing *E. coli*, were reported in several studies[12] as an important cause of community onset urinary tract infections[13] , as well as blood stream infections[14]. Multi-drug resistant *E. coli*[15,16] and ESBL producing *Enterobacteriaceae* have been reported from different hospitals in Sudan[17,18]. Recently, Musa has demonstrated predominance of *bla*CTX-M gene among ESBL producing *E.coli* and *Klebsiella spp* from Sudan[19]. We have undertaken this study in order to determine the prevalence and mechanism of antimicrobial resistance among Enterobacteriaceae from Sudan.

MATERIALS AND METHODS
Bacterial strains

This study was carried out in two major tertiary care referral hospitals in the State of Khartoum (Omdurman and Khartoum teaching hospitals) in order to determine the prevalence and mechanisms of antimicrobial resistance among Enterobacteriaceae from Sudan. Clinical isolates were collected from patients and healthy individuals during the period 2003-2007. Two hundred clinical isolates, were obtained from each hospital Microbiology Laboratory and 121 normal fecal strains from inpatients and 500 fecal strains from healthy individuals in the community were included in the study. All 1021 bacterial strains were identified to the species level by standard microbiology methods[20] and confirmed by API-20E and were stored at -20°C in Cryopreservers until tested.

Antibiotic susceptibility testing

All 1021 bacterial isolates were tested for antibiotic susceptibilities by disc diffusion according to BSAC standardised methods using Iso-Sensitest agar for 18 antimicrobials (Ampicillin, Cephalexin, Cefuroxime, Cefotaxime, Ceftriaxone, Ceftazidime, Meropenem, Co-amoxyclav, Nalidixic acid, Ciprofloxacin, Streptomycin, Gentamicin, Amikacin, Chloramphenicol, Trimethoprim, Co-trimethoprim, Tetracyclin and Rifampicin) (Oxoid Ltd, Basingstoke, UK)[21]. Minimum inhibitory concentrations (MICs) were determined to Ampicillin, Cefotaxime, Ceftazidime, Cefuroxime, Co-amoxiclav, Chloramphenicol, Ciprofloxacin, Gentamicin, Tetracycline and Trimethoprim. Following the BSAC agar incorporation standardised technique[22].

ESBL-Screening test

Bacterial strains resistant to ceftazidime and/or cefotaxime (113) were further screened for the production of Extended Spectrum Beta-Lactamases (ESBLs), according to BSAC guide lines[23]. Screening test was carried out using cefpodoxime (10µg) and cefoxitin (30µg) (Oxoid) antibiotic discs. Bacterial strains that were resistant to cefpodoxime were considered as ESBL producers while resistance to Cefoxitin indicates Amp-C production[23].

ESBL-Confirmatory test

The Bacterial strains (113) were then subjected to the confirmatory test by the combined disc method. Three sets of antibiotic discs (Mast Laboratories Ltd, Bootle, Merseyside, UK) were used[23].

Plasmid analysis and conjugation studies

Conjugal transfer of resistance determinants was performed by broth culture with *E. coli* K12 as a recipient. After 24 hours incubation, mating mixtures were plated onto agar supplemented with nalidixic acid (30µg/ml) and ampicillin (10 µg /ml). Plasmid DNA was prepared from donors and transconjugants using

a commercial kit (Plasmid Mini Kit, Qiagen GmbH, Hilden, Germany).

PCR amplification and sequencing

DNA for PCR was extracted by boiling of suspended bacteria on sterile water for 10 minutes. PCR was carried out for the identification of the *bla* genes coding for extended spectrum β-lactamases (TEM, SHV, and CTX-M) and for Amp-C Beta-Lactamases. PCR products were detected by electrophoresis on 1.5% (w/v) agarose gels visualized under UV light. Sequence determination of amplicons was performed using the respective PCR primers for both directions using ABI 3100 automated sequencer (Warrington, UK) and compared on BLAST.

Statistical Analysis

Results were analysed using SPSS software program.

Ethics approval and consent to participate

Not applicable, all study samples (isolates) were obtained from microbiology laboratories.

RESULTS

Isolates included in this study were resistant to ampicillin (MICs>64), first and second generations cephalosporins (MICs>4) and to expanded-spectrum cephalosporins (MICs>4), in addition to three or more antimicrobials such as gentamicin (MICs>4), ciprofloxacin (MICs>4),

trimethoprim (MICs>8), tetracycline (MICs>4) and co-amoxyclav (MICs>64). All isolates were sensitive to meropenems.

Screening of all the isolates (113) with Cefpodoxime showed that resistance among 111(98%) of the isolates.

Over all 106(94%) isolates were confirmed by the Combined disc method for ESBL production while 7(6%) isolates were tested negative (Table 1).

ESBLs-producing strains

For the characterization of ESBL only multidrug resistant strains were resistant to cefotaxime or/and ceftazidime antimicrobials were included. The total number was One-hundred and Thirteen strains (113). Consisted of 103 (91%) clinical isolates and ten stool commensals (9%), eight of which were from hospitalized patients and two were from healthy individuals (table-2). *bla* genes were detected by PCR in 94% of the isolates (106/113), it includes different enterobacterial species comprising *E.coli, Klebsiella pneumoniae*, *Proteus spp*, *Enterobacter spp*, *Salmonella spp* and few others. Some isolates were found to possess more than one type of ESBL as well as the prototypic TEM-1 β-lactamase enzyme (Table 3).

Table 1. Comparison between cefpodoxime screening test and the combined disc test results.

	Combined disc +ve	Combined disc -ve	Total
Cefpodoxime resistant	106	5	111
Cefpodoxime sensitive	0	2	2
Total	106	7	113
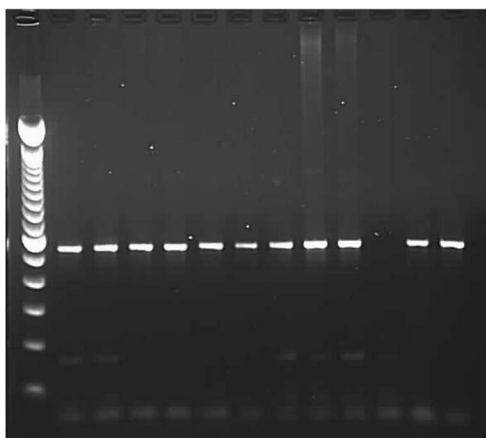
Fig. 1. PCR results of *bla*CTX-M positive isolates.
Lane 1: Lambda DNA of a multiple 100bp size marker.
Lanes 2 to 10: *bla*CTX-M positive isolates.
Lane 11: negative control.
Lane 12, 13: positive controls.
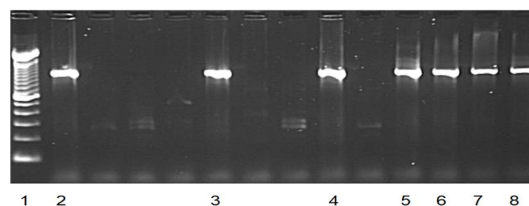
Fig. 2. PCR results of *bla*SHV positive isolates.
Lane 1: Lambda DNA of a multiple 100bp size marker.
Lanes 2, 3, 4, 5, 6 and 7: *bla*SHV positive isolates.
Lane 8: positive control.

Table 2. Number of bacterial isolates included in the study

Bacterial isolates		Number
Clinical pathogens		103 (91%)
Stool commensals	Inpatients	8
	Community	2 10(9%)
Total		113

Table 3. Distribution of *bla*genes in different enterobacterial species detected by PCR.

*bla*gene	*E.coli*	*Klebs*	*Proteus*	*Enterob*	*C-freu*	*Proved*	*Morg*	*Sum*
CTX-M	50	30	3	4	2	0	1	90
SHV	11	21	0	7	0	0	0	39
TEM	46	32	3	5	2	1	1	91

Table 4. Sequencing results for *bla*SHV-positive isolates

SHV-TYPE	Total No. isolate	ORGANISMS		
		E.coli	*Klebsiella*	*Enterobacter*
SHV-1	12	6	6	0
SHV-1a	4	0	4	0
SHV-5	4	0	3	1
SHV-5a	7	1	2	4
SHV-11	3	2	1	0
SHV-12	3	1	1	1
SHV-26	2	0	2	0
SHV-28	2	1	1	0
SHV-38	2	0	2	1

DNA-sequencing results revealed CTX-M-15 was detected among all CTX-M positive isolates except for an *E.coli* isolate which was CTX-M9 producer. In all 39(34.5%) of the isolates tested for ESBLs were *bla*SHV positive, only 11(28%) encode for ESBLs (Table 4). DNA-sequencing results for *bla*TEM positive isolates revealed *bla*TEM-1 prototype.

Resistance transfer to the expanded-spectrum cephalosporins was achieved among all the tested isolates, as well as the resistance to the other antimicrobial groups which was also co-transferred to the recipient organisms.

DISCUSSION

We have undertaken this study in order to find out the Prevalence of ESBL-producing *Enterobacteriaceae* among hospitalized patients and healthy subjects in the community and to characterize ESBL genes, as the first study of its kind in Sudan. The prevalence among *Klebsiella spp.*, *E.coli*, *Proteus spp.* and *Morganella morganii* isolates from hospitalized patients was exceptionally high when compared to that from the community. Similar high ESBL prevalence among *Enterobacteriaceae* in Sudan was reported later by Musa and Yousif[18,19]. ESBL-producing organisms, mostly *Klebsiella spp.* and *E.coli*. have been detected widely worldwide, but a few years later ESBL production by other Enterobacterial species have been reported[24,25,26]. The most frequently encountered ESBL genes were CTX-M. This is consistent with the findings of Musa and Yousif from Sudan. However, our study was more detailed as we characterized CTX-M genes further and found that they are consistent mostly of CTX-M15.

The CTX-M15 enzyme belongs to the CTX-M-1 cluster and is derived from CTX-M-3 by a single Asp-240-Gly substitution[27] and has the highest catalytic activity against ceftazidime among all the CTX-M clusters, this explains the high resistance level to Ceftazidime among this group of our isolates. CTX-M15 has been reported from different parts of the world, it has been described in many genera of enterobacteriacae across the world, and is the most reported variant in much of Europe[28]. We identified CTX-M15 in a number of Enterobacterial species, including *E.coli*, *Klebsiella spp.*, *Proteus spp.*, *Enterobacter spp.*, *Citrobacter freundii* and *Morganella morganii*. All these strains were resistant to multiple antimicrobial agents. CTX-M15 variant was also detected in fecal commensal isolates in this study, two *E.coli* isolates from the community and six isolates including *E.coli* and *Klebsiella spp* from the fecal samples of hospitalized patients. The CTX-M-15 variant which occurs mainly in *E.coli* seems to be epidemic in

most countries. It is reported from many countries within the African continent with other ESBL-enzymes. From Tanzania in a pediatric hospital[29] and an intensive care unit[30], from Cameroon[31]. Another report from sub-Sahara Africa was from Central African Republic[32], Malawi[26]. And also North Africa from Tunisia[33].

Conjugation studies from this study have shown the possibility of horizontal gene transfer between various enterobacterial species carrying resistance genes to many antimicrobial groups including β-lactams. This could explain the acquisition of CTX-M15 and other ESBLs genes by different species of enterobacteriaceae. Other studies has reported similar findings, from Nigeria[34], and Malawi[26], showed that ESBL genes were associated with large conjugative plasmids that carry other resistance determinants and were responsible of resistance transfer between different bacterial species.

Only one isolate (*E.coli*) from this study was encoding *bla*CTX-M9, reported and sequenced in an *E.coli* from Spain[35] and also later reported in an *E.coli* causing community acquired UTI infections[36].

Variable *bla*SHV genes encoding ESBLs have been detected in this study from clinical as well as commensals. *bla*SHV5 and *bla*SHV5a were detected in 9.7% of the ESBL producing isolates, both genes were isolated from *Enterobacter spp* and from *K. pneumoniae*. SHV5a was detected in an *E.coli* isolate from the community commensal collection as well. SHV5 has been reported by different studies from Thailand[37], Poland[38], Australia[39] and was isolated from a healthy student in Lebanon[40]. From the African continent SHV-5 has been reported from South-Africa in two different studies[41,42].

*bla*SHV12 was detected in two (2.7%) isolates (*Klebsiella pneumoniae* and an *Enterobacter spp*); both isolates were CTX-M15 producers. *blaSHV12* is the most commonly identified genotype worldwide. within the African continent SHV-12 was reported from Tanzania[29,31], Senegal[43], Cameroon[32] and from the Central African Republic[33].

blaSHV genes with specific hydrolytic activities to certain antimicrobials were also detected. SHV-38 was detected in *Klebsiella* and an *Enterobacter* isolates, its hydrolytic activity includes only ceftazidime and imipenem. It has been reported from France[45]. SHV-26 was also detected in this study in two *K. pneumoniae* isolates, they were resistant to co-amoxyclav. SHV-26 is a β-lactamase with reduced susceptibility to clavulanic acid that renders bacteria intermediately resistant to an inhibitor containing β-lactam. Other *blaSHV* genes were also reported by this study including *bla*SHV-28 among two isolates *K. pneumoniae* and *E.coli*.

And SHV-11 which infrequently reported and is not considered as an extended spectrum β-lactamase[44]. The prototypes to TEM and SHV-1/SHV-1a β-lactamase enzymes were detected as well among the majority of the isolates.

This part of the study has illustrated a great variety of beta-lactamases genes, which reflects the magnitude of the problem of antimicrobial resistance in Sudan, and the wide misuse of the expanded-spectrum beta-lactams. Over all 69% of the isolates were carrying *bla*CTX-M gene as well as *bla*SHV gene. 31% were carrying only *bla*SHV genes and were all ESBL-producers.

Detection of CTX-M15 and the variety of SHV enzymes among the majority of isolates would render all third generation cephalosporins ineffective for the treatment of enterobacterial infections. Finding of Amp-C within these isolates was an alarming sign of the development of resistance to cephamycins which have not yet been introduced into clinical practice in Sudan. All the above would limit the treatment options for the physicians who would be faced with only one option. Imipenem has recently been introduced in The Sudan. However all the isolates showed complete susceptibility to it and it could be one of the options for the treatment of multi-resistant infections. However already a resistance gene (*bla*SHV-38) that confers resistance to ceftazidime as well as to imipenems has been detected among these isolates.

Resistant bacteria may also spread and become major infection-control problems. Not only within health-care institutions, but also in the community. Clinically important bacteria, such as extended-spectrum β-lactamase (ESBL)-producing *E.coli*, are increasingly observed in the community as has been reported in several studies[37,46].

In this study, multiple drug resistance to three or more antimicrobials is highly prevalent

among the clinical isolates found in variable enterobacterial species. A wide range of gram-negative bacterial species were found to be harboring resistance to the majority of commonly used β-lactam antibiotics as well as to other antimicrobial groups. Infection with multi-resistant pathogens will complicate the treatment of nosocomial gram–negative bacterial infections within the Sudanese hospitals and will increase the mortality rates due to ineffective treatment of patients complicated by the limited options available for the doctors. This will result in further nosocomial dissemination of multi-resistant isolates due to selective pressure applied by the use of these drugs.

Compliance with Ethical Standards

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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