Citation: Abd El Rahman M. Abd El Rahman. Detection of CTX-M, TEM and SHV Genes in Gram-negative Bacteria isolated from Nosocomial Patients at Port Sudan Teaching Hospital (Sudan). 2017, 2 (3). ajmsc.info

Detection of CTX-M, TEM and SHV Genes in Gram-negative Bacteria isolated from Nosocomial Patients at Port Sudan Teaching Hospital (Sudan)

Abd El Rahman M**.** Abd El Rahman
University of Khartoum, Khartoum, Sudan

Abstract

Background: Bacteria from clinical and non-clinical settings are becoming increasingly resistant to conventional antibiotics and broader information-control problem. Patients admitted to hospitals for the treatment of resistant bacterial infections are adding to the already too high costs of healthcare and are a source of resistant bacteria and/or resistance-encoding genes.
Objective: To detect CTX-M, TEM and SHV genes in Gram-negative bacteria isolated from nosocomial patients at Port Sudan Teaching Hospital (Sudan).
Materials and methods: The present study was a descriptive cross-sectional study conducted between September 2011 to September 2012 from hospitalized patients suspected of having Gram negative diseases at Port Sudan teaching hospital in red sea state. Isolation and identification of pathogenic bacteria were carried out following standard laboratory procedures. All isolates were tested to 14 types of common antimicrobial uses. Identification of extended spectrum beta lactamase (ESBL) production was performed by the double disk synergy test and double disk diffusion test. ESBL positive phenotypically were tested for the presence of ESBL encoding genes using PCR with specific primers for the detection of CTX-M, TEM and SHV genes, then the amplicons were sequenced to characterized gene content. Collected data were analyzed by using Statistical Package for Social Sciences (SPSS; Version 20). P value <0.05 were considered statistically significant.
Results: the results showed 198 types of Gram negative bacteria isolated. The major isolates was *E.coli* (89/44.9%) and the least Gram negative organism isolated were *Klebsiella oxytoca, Serratia marcescens, Morganella morganii, Salmonella para typhi A* and *Citrobacter koseri* (1/0.5%). This difference was statistically significant (*p* 0.00). ESBL producing bacteria was 44.4%, and was mostly *E.coli* 63.6%. The maximum sensitivity was seen for amikacin (96.6%) the different was statistically significant (*p* 0.00). While the maximum resistance was seen against ceftriaxone, ciprofloxacin and tetracycline (100%). The presence of CTX-M, TEM and SHV genes was confirmed in 65/ 88 (73.9%) of the isolates. The ESBL genes were detected in

47 *Escherichia coli*, 14 *Klebsiella pneumoniae*, 2 *Proteus mirabilis*, 1 *Serratia odotifera* and 1 *Enterobacter sakasaki*.

Conclusion: The study concluded prevalence of ESBL among Gram-negative bacilli at Port Sudan teaching hospital in red sea state, in Sudan was found to be higher compared with other countries. Among isolated organisms the commonest genes was CTX-M gene followed by TEM gene, while the least one was SHV gene. Further surveillance need to determine other genes among isolated pathogens.

Key words: CTX-M, TEM and SHV genes, Gram-negative bacteria, Nosocomial infection.

Introduction

Multiplex-PCR was first described in 1988 as method to detect deletions in the dystrophic gene. By targeting multiple genes at once, additional information may be gained from a single test run that otherwise would require several times, more reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction and amp icon size i.e., there base pair length should be different enough to form distinct bounds when visualized by gel electrophoresis[1].

The prevalence of ESBLs among clinical isolates varies from country to country and from institution to institution. In the United States, the occurrence of ESBL production in *Enterobacteriaceae* ranges from 0to 25% depending of the institution, with the national average being 3%[1].

Resistance to 3rd generation cephalosporins due to acquisition and expression of extended spectrum β-lactamases (ESBLs) enzymes among Gram-negative bacteria is on the increase and the presence of ESBLs producing organisms has been reported to significantly affect the course and outcome of an infection management worldwide especially in already overburden health systems in developing countries. ESBL producing organisms are reported to account for a significant proportion of intensive care infections. Problems of ESBLs have led to limited as well as expensive treatment options, and have impacted negatively on clinical outcomes. Nosocomial infections due to ESBL producing organisms have been known to cause high mortality, so the aim of this study was to determine the existence and to describe phenotypic and genotypic characteristics of extended- spectrum β-lactamase-producing gram-negative bacilli isolates from different clinical specimens of patients attending to port Sudan teaching hospital, and to heighten awareness of these in reducing treatment failure, limiting their introduction into hospital and preventing the spread of these emerging pathogens within the community.

During the last few decades, many pathogenic bacteria have evolved and developed antimicrobial resistance. The emergence and increase of antimicrobial resistance in pathogenic bacteria poses an important challenge in human health due to higher risk of morbidity and mortality because of delayed or incorrect treatment with effective antimicrobial therapy[2].

Determination of phenotypic resistant is a useful guide to proper treatment of microbial infection

since they can determine significant differences and shifts in susceptibility to various antimicrobial agents. In addition, genetic studies such as CTX-M, TEM and SHV genes by molecular methods that include characterization of resistance determinants are necessary to understand fully the spread and development of antimicrobials by providing information on the transmission of resistant strains. Their persistence in the bacterial population and the resistance mechanism involved[3].

The World Health Organization (WHO) identified a lack of information concerning microbial genetics and ecology in antimicrobial resistant bacteria as a gap in the current knowledge[3]. The study may advise on the appropriate antimicrobials needed for treatment of multi-resistance infections problems and may minimize the use of antimicrobials for nosocomial infections. Further it may assist in the control of bacteria expressing ESBL and advice on practical measures to limit its spread and enhance its elimination.

The study also may monitor and reflect the magnitude of ESBL resistance problem in Red Sea State. It may also encourage researchers to look for possible sources of ESBL bacteria, and to test for ESBL resistance in a wide range of pathogenic organisms.

Materials and methods

This was a case-finding hospital and laboratory-based study. It was a descriptive, cross-sectional, qualitative study aiming to screen patients with nosocomial, bacterial infections at Port Sudan Teaching Hospital (Sudan). The study was conducted between September 2011 and September 2014. Target population was patients suspected of having Gram negative bacterial infections. Inclusion criteria were patients suffering from Gram negative bacterial infections and hospitalized at least for at least three days. Exclusion criteria were patients with non-bacterial infections. Complete information regarding risk factors was handed to all patients under the study and no concealment what so ever. Confidentiality of information obtained from patients was maintained. Valid consent of the patients under the study was obtained. Results of samples collected were donated to all patients included in the study and some sample results were dispatched to physicians for treatment prescription. Approval to collect the samples was obtained from Port Sudan Teaching Hospital administration. Permission to conduct the study was granted by University of Khartoum (Sudan). Collected data of patients, antimicrobial susceptibility of gram negative bacterial isolates and their molecular characterization were analyzed, using the Statistical Package for Social Sciences (SPSS; Version 20). Data were arranged and entered into SPSS according to program guidelines. Means and level of the significant differences between every two variables were tested using independent samples T-Test and one-way Analysis of Variance (ANOVA). All p-values < 0.05 were considered statistically significant.

Sampling was a non-probability purposive type. Sample frame was the bacterial infections patients at Port Sudan Teaching Hospital. Sample strategy was a convenience type where patients were chosen on the basis of accessibility. Sample size was selected randomly due to unavailability of prevalence data. 400 different samples were collected from patients with bacterial infections.

A questionnaire was used to collect personal, demographic and clinical data from laboratories registration books in the selected hospitals. The data included age, gender, hospital unit and site of infection. Bacterial isolates were obtained after investigating various clinical specimens including urine, blood, wound swab, ear swab and miscellaneous body fluids. Urine specimens and miscellaneous body fluids were collected in sterile plastic containers and were transported to the microbiology laboratory to be processed immediately for detection of pathogenic Gram-negative bacteria. Blood samples were extracted under aseptic conditions and transferred immediately to sterile blood culture bottles containing brain heart infusion broth. Ear and wound swabs were collected, placed in transport media, and investigated as soon as possible.

Isolation, identification, and antimicrobial susceptibility testing of gram-negative bacilli were carried out in a systemic way according to standard microbiological methods[4]. Screening of nosocomial isolates for strains expressing an extended-spectrum beta-lactamases (ESBLs) was performed by detection of reduced zones of inhibition around the third generation cephalosporins discs as recommended by the Clinical and Laboratory Standards Institute[5]. Detection of ESBLs strains was confirmed by the double disc synergy test (DDST) and the double disc diffusion test (DDDT); and then they were further genotyped by PCR technique.

Isolation of Gram-negative bacteria from urine specimens was done by culturing directly onto CLED, Mac Conkey and blood agar plates (Oxoid, Basingstoke England), using sterile nichrome wire calibrated loop. While the culture of body fluids specimens was done by cultivation onto Mac Conkey and blood agar plates. The isolation of Gram-negative bacteria from ear and wound swabs was done by inoculating onto Mac Conkey and blood agar plates[4].

All cultured plates were incubated aerobically for 24 hours at 37°C and were identified on the basis of cultural characteristics, gram stain, conventional biochemical tests, and API 20E identification system (BioMerieux, Marcy-I'Etoile, France) Culture plates which yielded more than two organisms per specimen were excluded from the study.

The API identification system API 20E uses 23 standardized and miniaturized biochemical tests in a single strip. The test was performed as per manufacturer's instructions. Identification was performed using the database of the analytical profile index (API cat. No. 20190-BiMerieux); where each code number indicates a bacterial strain[6].

After full isolation and identification, 2 ml of an overnight culture was subcultured in tryptic soy broth, mixed with 1 ml of sterile 20% glycerol solution, and stored at -20 ^{0}C for later antimicrobials susceptibility testing and determination of nosocomial specimens isolates expressing an extended-spectrum beta-lactamase (ESBL) phenotype, as well as screening for genotypes by PCR amplification.

Antimicrobial susceptibility testing of Gram negative bacteria isolates was performed on Mueller-Hinton agar plates (Oxoid, Basingstoke England) using the Kirby-Bauer disk diffusion method following the CLSI recommendations.14 antimicrobial agents were tested (Liofilchem Co. Italy), namely amikacin (30 µg), amoxicillin (10µg), amoxicillin-clavulanic acid (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), nalidixic acid (30 µg), nitrofurantoin (50 µg), tetracycline (30 µg), tobramycin (10 µg) and trimethoprim-sulfamethoxazole (25 µg).

Standardized inocula conforming 0.5 McFarland standard turbidity were employed. E. coli ATCC 25922 (American Type Culture Collection) was used as control and tested each time when susceptibility testing was performed. Test results were only validated when inhibition zone diameters of the control strain was within the performance range in accordance to CLSI guidelines[5].

Detection of β-lactamase activity was performed by the nitrocefin method. Nitrocefin is a chromogenic, cephalosporin substrate routinely used to detect the presence of beta-lactamase enzymes produced by various Gram-negative bacteria. Nitrocefin contains a beta-lactam ring which is susceptible to beta-lactamase mediated hydrolysis. Once hydrolyzed, the degraded nitrocefin compound rapidly changes color from yellow to red. Although nitrocefin is considered a cephalosporin, it does not appear to have antimicrobial properties. This method is recommended by the National Committee for Clinical Laboratory Standard (NCCLS) and the World Health Organization (WHO). The working solutions of nitrocefin used in this method were within 0.5-1.0mg/mL[7].

ESBL screening test was carried out on Muller Hinton agar plates using the Kirby-Bauer disk diffusion method. Antibiotics discs used (Liofilchem Co. Italy)were: cefotaxime (30µg), ceftazidime (30µg), aztreonam (30µg), cefpodoxime (10µg) and ceftriaxone (30µg). The size of the inhibition zones was compared with zone diameter recommended by CLSI[5], screening criteria. The double disc synergy test (DDST) was carried out on Muller-Hinton agar plate seeded by bacterial suspension. A disc containing the amoxyclav (amoxicillin 20 µg plus clavulanic acid 10 µg) was placed at the center of the Muller-Hinton agar plate. Four discs (cefepime (30µg), ceftazidime (30µg), cefotaxime (30 µg), and aztreonam (30 µg) were placed around amoxyclav disc at a distance of 25mm center to center (26). Read after overnight incubation. An extension of the inhibition zone towards the disc containing the amoxyclav indicates an ESBL activity (Fig. 1).

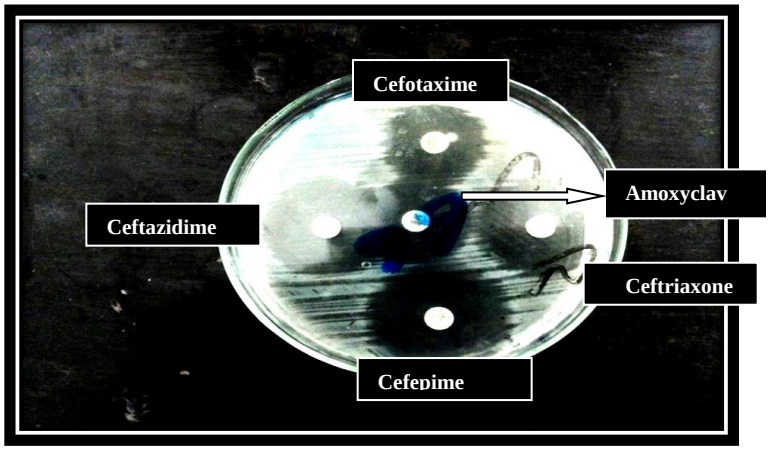

Fig. 1: Double-disk synergy test (DDST) showing ESBL activity.

Phenotypic confirmatory test was performed using the disk diffusion test (DDT). Antibiotics selected were ceftazidime (30 µg), cefotaxime (30 µg), and cefepime(30 µg). Each disk was placed on Muller-Hinton agar plates with and without clavulanic acid (10µg). A difference of $\geq$ 5mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disk was considered to be phenotypic confirmation of ESBL production[5]. *E.coli* strain ATCC 25922 was used as a negative control and *Klebsiella pneumoniae* ATCC 700603 was used as a positive control.
The plasmid DNA was extracted using DNA extraction kits (iNtRon Biotechnology, Seongnam, Korea). Procedure adopted was as per manufacturer's instructions. The concentration of the extracted DNA was measured according to the instructions of the manufacturer using a spectrophotometer (Bio-Eppendrof). An aliquot of the DNA was electrophoresed on 1.5% agarose gel to ensure the purity of the extracted DNA. The forward and reverse specific oligonucleotide primers used in PCR for detection of CTX-M, TEM and SHV genes were:

- 5- SCS ATG TGC AGY ACC AGT AA-3 and 5- CCG CRA TAT GRT TGG TG-3 for CTX-M gene[7].
- 5- TCG GGG AAA TGT GCG CG-3 and 5- TGC TTA ATC AGT GAG GCA CC-3 for TEM gene[7].
- 5-GGT TAT GCG TTA TAT TCG CC-3, 5-TTA GCG TTG CCA GTG CTC-3 for SHV gene[7].

All isolates were screened for the resistance genes *ble* CTX-M, *ble* TEM, *and ble* SHV by polymerase chain reaction (PCR), using their specific primers. The amplification was done using Convgys® td Peltier thermal cycler (Gmbh and Co.KG, Germany). Prepare a PCR mixture reaction of 25ul volume containing 5ul master mix of Maxime RT Premix kit, 0.6 forward primer, 0.6 reverse primer, 2ul plasmid DNA and 16.8ul deionized sterile water. This mixture was subjected to initial denaturation step at 94° C for 5min, followed by 30 cycles of denaturation at 94° C for 45 seconds, primer annealing at 57° C for 45 seconds, and followed by step of elongation at 72° C for 60 seconds. The final elongation step was at 72° C for 5min[7].
The genes amplification products were detected by gel electrophoresis. This was carried out by loading 5µl of each amplified product on 1.5% agarose gel in 1X TBE buffer containing (2.5ug/ml)ethidium bromide (Qiagen, Germany). The DNA ladder marker with size 100.0 Kb was used as standard molecular weight for determining the size of these products. The gel electrophoresis run was 100 volts for 30 minutes. The bands were visualized under U.V transilluminater (Uvite-UK).Positive and negative controls were run with each batch. DNA extracted and sequencing from known CTX-M, TEM and SHV gene was used as a positive control. A sample with no DNA template was used as a negative control.
Sequencing of CTX-M, TEM and SHV genes was performed to determine the nucleotide sequence of the gene DNA. DNA purification and standard sequencing was performed for both strands of all genes. The samples were sealed in sterile Eppendrof tubes and sent for DNA sequencing by Macrogen Company (Seoul, Korea).

The sequence chromatogram was analyzed by Finch TV program. The nucleotides sequence of the CTX-M, TEM and SHV beta-lactamases genes were checked for sequence similarities using nucleotide BLAST[8] (29). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using Phylogen software program[9] (74).

Results:

A total of four hundred clinical specimens were obtained from patients attending Port Sudan Teaching Hospital; during the period from March 2011 to July 2012. Out of these 400 specimens 298 (74.5%) yielded a significant growth. 198 (66.4%) of the organisms isolated were Gram-negative bacilli, 70 (23.5%) were Gram-positive bacteria, 10 (3.4%) were yeasts, and 20 (6.7%) were mixed growth. From the 198 Gram-negative bacilli recovered, 110 (55.6%) were urine specimens, 67 (33.8%) were wound specimens, 9 (4.5%) were ear swabs, one (0.5%) was a blood specimen, and 11 (5.6%) were other body fluid specimens. *Escherichia coli* was the commonest (89/44.9%) organism isolated; while *Salmonella paratyphi A*(1/0.5%) and *Citrobacter koseri*(1/0.5%) were the least Gram-negative bacilli recovered.
Among the 198 infected Gram-negative bacilli patients, 125 (63.1%) were females and 73 (36.9%) were males. This gender difference was not significant (p = 0.896).Patients enrolled in the study were divided into three age groups: less than 12 years old, 13-49 years old and more than 50 years old. The highest frequency of isolates (99/50%) was in the age group 13-49 years, and the lowest frequency of isolates (5/2.5%) in the age group of less than 12 years.
On the other hand, 88 ESBL-producing Gram negative bacilli were subjected to PCR testing. From these the existence of CTX-M, TEM, SHV genes was detected among 65 (73.9%) ESBL producers. The most frequent gene detected was CTX-M gene (53/60.2%), followed by TEM gene (22/25%), and then SHV gene (13/14.8%).This difference in the frequency of ESBL genes was found significant (p = 0.00). CTX-M gene was not detected among strains of *Serratia odotifera* and *Enterobacter sakasaki,* TEM gene was not detected among strains of *Proteus mirabilis,* and SHV gene was not detected among strains of *Serratia odotifera* (Table 1).

Table (1): CTX-M, TEM and SHV genes detected among ESBL producers

Genes detected	*Escherichia coli*	*Klebsiella pneumoniae*	*Proteus mirabilis*	*Serratia. odotifera*	*Enterobacter sakasaki*
CTX-M	44(65.7%)	7 (46.7%)	2(66.7%)	0 (0.0%)	0 (0.0%)
TEM	14(20.9%)	6 (40%)	0 (0.0%)	1 (100.0)	1 (50.0%)
SHV	9 (13.4%)	2 (13.3%)	1(33.3%)	0 (0.0%)	1 (50.0%)

Out of the 47 *Escherichia coli* isolates, 2 strains (4.3%) had harbored all three ESBL genes. While out of the 14 *Klebsiella pneumoniae* isolates, one strain (7.1%) had harbored both CTX-M and SHV genes.
As shown in Table (2), the highest resistance rate of ESBL-producing Gram-negative bacilli was observed against ceftriaxone (100.0%), tetracycline (100.0%), and ciprofloxacin(100.0%). The highest antimicrobial activity of ESBL-producing organisms was observed with amikacin (96.6%). ESBL-producing Gram-negative bacilli isolates were significantly more resistant to the antibiotics tested as compared with ESBL non-producing isolates ($p< 0.05$).

Table (2): Antimicrobial susceptibility pattern of the bacterial isolates

Antibiotic	Sensitive (%)		Resistant (%)		p value
	ESBL producer	Non-ESBL producer	ESBL producer	Non-ESBL producer	
Amikacin	96.6	96.4	3.4	3.6	0.932
Amoxicillin	4.5	1.8	95.5	98.2	0.268
Amoxyclav	1.1	94.5	98.9	5.5	0.000
Ceftazidime	49.0	99.1	51	0.9	0.000
Ceftriaxone	0.0	70.9	100	29.1	0.000
Cefuroxime	1.1	12.7	98.9	87.3	0.002
Chloramphenicol	63.6	100	36.4	0.0	0.000
Ciprofloxacin	0.0	72.7	100	27.3	0.000
Gentamicin	29.5	99.1	70.5	0.9	0.000
Nalidixic acid	1.1	45.5	98.9	54.5	0.000
Nitrofurantoin	50	100	50	0.0	0.000
Tetracycline	0.0	41.8	100	58.2	0.000
Tobramycin	53.4	100	46.6	0.0	0.000
Co-trimoxazole	1.1	22.7	98.9	77.3	0.000

Out of the 47 *Escherichia coli* isolates, 2 strains (4.3%) had harbored all three ESBL genes. While out of the 14 *Klebsiella pneumoniae* isolates, one strain (7.1%) had harbored both CTX-M and SHV genes.

Furthermore, of the 198 Gram-negative bacilli isolates, 94 (47.5%) had produced ESBL activity by the double-disk synergy test, and 88 (44.4%) had produced this activity by the confirmatory double-disk diffusion test. This difference was statistically significant ($p = 0.00$).*Escherichia coli* was the most common ESBL producer on testing by both double-disk synergy test and double-

disk diffusion test. Also wound swabs were found to be the commonest source (34/67:50.7%) of ESBL-producing isolates (Table (3).

Table (3): Sources of ESBL-producing isolates

Sample	Urine	Wound swabs	Ear swabs	Body fluids
Isolates	ESBL producer	ESBL producer	ESBL producer	ESBL producer
Escherichia coli	39/110 (35.5%)	15/67(22.4%)	0/9 (0.0%)	2/11 (18.2%)
K. pneumoniae	5/110 (4.5%)	16/67 (23.9%)	0/9 (0.0%)	2/11 (18.2%)
Proteus mirabilis	3/110 (2.7%)	0/67(0.0%)	3/9 (33.3%)	0/11 (0.0%)
Serratia odotifera	0/110 (0.0%)	1/67(1.5%)	0/9 (0.0%)	0/11 (0.0%)
E. cloacae	0/110 (0.0%)	1/67(1.5%)	0/9 (0.0%)	0/11 (0.0%)
E. sakasaki	0/110 (0.0%)	1/67(1.5%)	0/9 (0.0%)	0/11 (0.0%)
Total	47/110 (42.7%)	34/67 (50.7%)	3/9 (33.3%)	4/11 (36.4%)

On sequencing, the CTX-M gene (No. 25) responsible for ESBL activity showed deletion of the amino acid adenine at position 31. Guanine had substituted the amino acid adenine (G-A) at position 49, compared to the wild-type reference sequence. The CTX-M gene (No. 24) also showed a substitution of the amino acid adenine by guanine (G-A) at position 49 compared to the wild-type reference sequence.
Chromatogram sequence alignment for TEM gene (No.27) showed substitution of amino acid thymine to adenine (T-A) at position 5, and substitution of amino acid thymine and cytosine to cytosine and adenine respectively at position 14, 15. This gene also showed deletion of amino acid guanine at position 47 as compared to the wild-type reference sequence. Chromatogram sequence alignment for SHV gene (No.32) showed deletion of amino acid adenine at position 9 compared to the wild-type reference sequence.

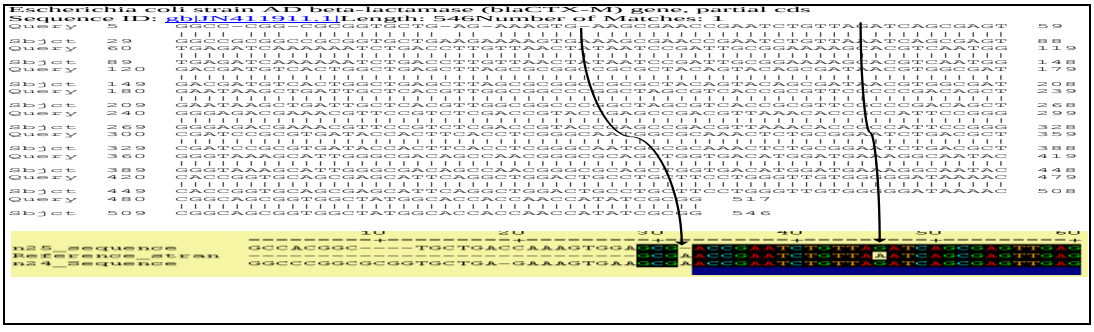

Fig. (1): Chromatogram sequence alignment for CTX-M gene.

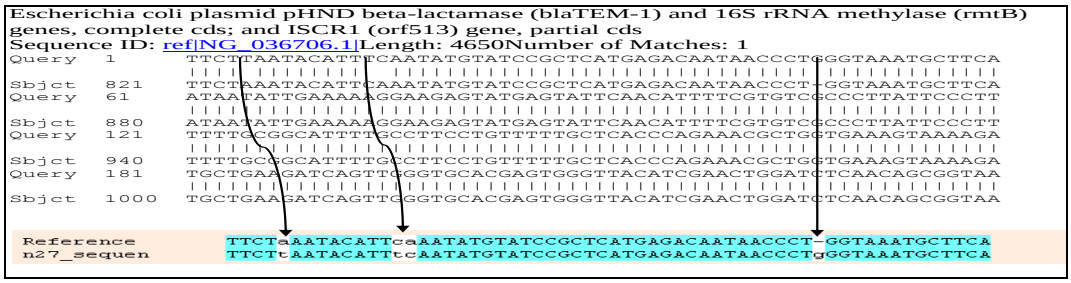

Fig. (2): Chromatogram sequence alignment for TEM gene.

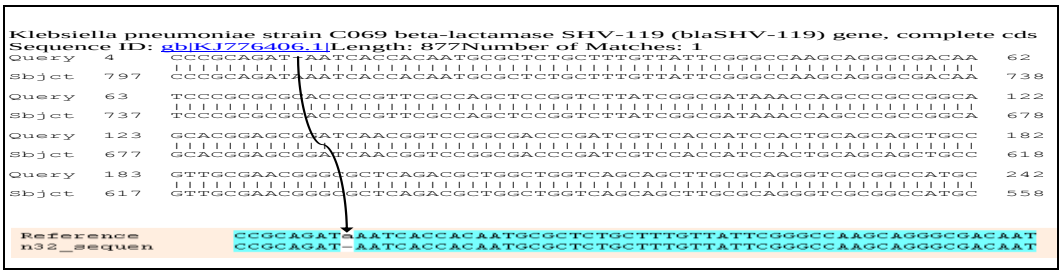

Fig. (3): Chromatogram sequence alignment for SHV gene.

Discussion

The present study demonstrated presence of ESBL-producing bacteria isolated from clinical specimens of patients in Port Sudan Teaching Hospital. Several factors contribute to the increased risk among hospitalized patients, namely the disruption of the normal gastrointestinal flora by administration of broad-spectrum antibiotics, colonization with hospital-associated strains, poor infection control practices, presence of indwelling devices including urinary catheters, and an immune suppressed state[10].

The correct detection of ESBL- producing bacteria is a challenge for the laboratories, requiring not only phenotypic tests but also genotypic tests for all genes associated with beta-lactamase production. These enzymes can be chromosomal or plasmid mediated. The gene code for the enzyme may be carried on integrons. The integrons help in the dissemination of antimicrobial drug resistance in health care settings[11].

According to the majority of epidemiological studies on ESBL, *Escherichia coli* and *Klebsiella pneumoniae* are the most common species implicated in this type of resistance. Also it has been observed that these two species were the most prevalence among ESBL-producing microorganisms, confirming with international multi-center studies. This is also in agreement with our study, where *Escherichia coli* was the most predominant (44.9%) Gram negative bacillus isolated, followed by *Klebsiella pneumoniae* (23.2%).ESBL detection is not routinely carried out in many microbiology laboratories of hospitals in developing countries, (80), as well

as in Sudan. The emergence of ESBL-producing strains creates a need for laboratory testing methods for detection of these enzymes among bacterial pathogens[12].

In the present study, ESBL-producers were detected phenotypically by DDST and the phenotypic DDDT confirmatory method. The DDDT test was compared with DDST and it was found to be an inexpensive alternative for the DDST, for the detection of ESBL producers. The DDST lacks sensitivity because of the problem of optimal disc space and the correct storage of the clavulanic acid containing discs. Therefore the Clinical and Laboratory Standards Institute (CLSI) recommended the use of DDDT for the phenotypic confirmation of the ESBL producers among Gram-negative bacilli. But these two tests had yielded an equal accuracy in the determination of ESBL production. These methods had been previously documented as accepted tests for detection of ESBL-producers[13]. Moreover, these two tests are simple to apply routinely along with antimicrobial susceptibility testing in hospitals.

Non-EBSL phenotypes were detected among 55.6% of the isolates studied. This figure is similar to that figure reported in Sudan[2], where the frequency rate of ESBL producers was 40%. However, this frequency is low compared to that reported in another study carried out in Khartoum where ESBL producers among Gram-negative isolates were 53%[14]. Also frequency rates lower than 60.9% were observed in Egypt[15]. However, the current study findings are similar to that obtained in Sudan[7] where Gram-negative isolates frequency rate was 45%. In *Pseudomonas aeruginosa*, the ESBL production was not detected. The possible explanation for this may be its resistance mechanism is mediated by the production of metallo-betalactamase, lack of drug penetration due to mutations in the porins, or due to the loss of certain outer membrane proteins and the efflux pump[16]. In the present study, wound exudates were found to be the most common source of ESBL-producing isolates (50.7%). This is in agreement with another study conducted in India, where 70% of ESBL-producing isolates was obtained from wound exudates. The occurrence of ESBL-producing isolates in wound infections may be associated with prolonged hospital stay in spite of persistent treatment with antibiotics in different combinations. The risk of acquisition of multiple resistant organisms from medical devices and hospital environment is inevitable[17]. In our study, the frequency rate of ESBL-producing strains in urine specimens is 42.7%. *E. coli* is the main causative agent of urinary tract infections; consequently there is a wide spread of antimicrobials prescription to combat such infections. ESBL are becoming an increasing problem in hospitals. The possible explanation for colonization with ESBL-producing organisms may be long-term antibiotic exposure, prolonged intensive care unit stay, nursing home residency, severe illness, frequent use of ceftazidime and other third generation cephalosporins, and instrumentation or catheterization maneuvers[15].

Therefore, measures should be directed towards infection control practices in hospital units in order to prevent the spread of ESBL strains from one patient to another. This can be carried out by ensuring healthcare professional practices, hand hygiene, cleaning medical equipment, and preventing colonization of the environment. The present study detected high resistance rates among ESBL-producing strains to the first line antimicrobial therapy such as amoxicillin, co-trimoxazole, tetracycline, nalidixic acid, ciprofloxacin and amoxicillin-clavulanic acid. Similar rates of resistance have been previously reported in Sudan[18], and other developing countries.

Cephalosporins such as cefuroxime, ceftriaxone and ceftazidime had been used to treat Gram-negative bacterial infections of various body sites. In this study, higher resistance rates were observed among our isolates against ceftriaxone (100%) and cefuroxime (98.9%). A similar study in Saudi Arabia also detected high resistance to ceftriaxone. The high frequency rate of resistance to third generation cephalosporins (notably to ceftriaxone) in this study is of great concern since it was found to be much higher than what reported in other parts of the world. A possible explanation for this high resistance might be due to un-appropriate use of these drugs, or due to the presence of extended spectrum β-lactamases enzymes (ESBL). It is therefore important to screen for ESBL in clinical isolates to prevent widespread of resistant isolates in hospitals. As reported worldwide, our isolates were found susceptible to aminoglycosides (notably amikacin, tobramycin) and chloramphenicol. Similar findings were reported in India[19], where most Gram negative bacilli were found to be susceptible to Amikacin (67.4%). The explanation for that may be amikacin is a very powerful drug, and used only in hospital settings and not as first-line therapy. Hence it has a lower selective pressure due to its restricted use. In our study, ESBL-producing isolates exhibited significant higher resistant rates to non-β-lactamase antimicrobials agents including fluoroquinolones, aminoglycosides, tetracyclines and co-trimoxazole, as compared to ESBL non-producing isolates. The possible explanation for this observation may be due to the fact that ESBLs are encoded on plasmids and can be mobile; hence they would be easily transmissible resistance gene elements for other antimicrobials from one organism to another. 26.1% of ESBL strains studied lacked CTX-M, TEM and SHV genes. This can be explained by the possible presence of other ESBL encoding genes in the bacterial isolates studied. Most genes were detected among *E.coli* (47/72.3%) and *K. pneumoniae* (14/21.5%) strains. In this context CTX-M gene scored highest frequency rate (53/60.2%). This result is in agreement with that reported by Quinteros *et al* in Argentina[20] and Al-Azawy *et al* in Sudan[2]. However, it has been reported that the proportion of CTX-M gene strains among ESBL-producing isolates had dramatically increased from 38.2% to 87% worldwide. Also this study showed that CTX-M gene was more commonly encountered among *E.coli* strains with a frequency rate of 25/47.2%. This finding is in-agreement with the reports of Sekar *et al*[21] in India, who detected a frequency rate of 44.4%. However this rate is low when compared with that reported by Eltayeb *et al* in Sudan[8], who reported a frequency rate of 65.2%. The TEM gene was the second ESBL gene among our isolates (25.0%). This finding is similar to that reported by Al-Azawy et al. in Sudan[2], who found a frequency rate of 22.2%. In our study the frequency rate of SHV gene was low (14.8%). This result is similar to that (16.7%) reported by Al-Azawy et al. in Sudan[2]. The frequency rate of ESBLs TEM-type and SHV-type detected in this study were (25.0%)) and (14.8) respectively. Currently, little interference is observed as regard detection of these genes in Sudan. However other studies elsewhere showed a frequency rate of TEM and SHV gene in *E.coli* strains as (46.4) and (11.2%) respectively (104). Some reports showed that most ESBLs were derivatives of TEM and SHV genes and there are now more than 90 TEM-types and more than 25 SHV-types of β-lactamases[19]. In this study the TEM genes were amplified from (20.9%) of ESBL producing *E.coli* strains and (40.0%) of

ESBL producing *K. pneumoniae*. The rate of TEM in *E.coli* was lower than (57.5%) that reported by Tasli and Bahar. The frequency rate of TEM-type β-lactamase produced by *K. pneumoniae* in this study (40.0%) was higher (31%) than that reported by Tasli and Bahar[22]. The result in this study showed that the prevalence rate of SHV-type ESBL among *E.coli* strains and among *K. pneumoniae* strains were 13.4% and 13.3% respectively. This result is higher than that obtained by Eltayeb et al[7], who reported a 6.5% frequency rate of SHV gene among *E.coli* strains. Similar result was reported by Al-Azaway, *et al*[2], who detected 16% of SHV gene among *K. pneumoniae*. The ESBL genes production in this study was less common among *Proteus mirabilis* (3.4%). This result is less than that (11%) reported by Al-Azaway, *et al*[2] in Sudan. The ineffective use of antibiotics and the possibility of spreading ESBL gene between Gram-negative bacilli by transferable genes will lead to overwhelming spread of ESBL producing isolates**.** In our study the sequence alignment revealed an insertion, deletion and substitution of amino acids to the different genes of CTX-M, TEM and SHV, at much conserved regions. This mutation may be the cause of ESBL activity, because this mutation is located at beta-lactamase/transpeptidase-like domain which is the penicillin-binding domain of PBPs, that function as DD-transpeptidases, its catalyze the final step of cell wall biosynthesis by cross-linking two strands of peptidoglycan, or DD-peptidases. Similar explanation has been suggested by Eltayeb *et al*[8].
Conclusion: *E.coli* and *K. pneumoniae* were the major ESBL producing pathogen among isolates of Gram-negative bacteria. The most common ESBL gene is CTX-M, while prevalence of SHV genes was very low. ESBL types CTX-M, TEM and SHV genes are predominant among Gram-negative bacilli isolates and ESBL producer strains carried one or more than CTX-M, TEM or SHV gene. Amikacin and chloramphenicol constitute the drugs of choice for treatment of most infections caused by ESBL producers.

References

1. Hayden M. J, Nguyen T M, Waterman A. and Chalmers, K. J. ‘‘Multiplex-ready PCR: a new method for multiplex SSR and SNP genotyping.’’ *BMC Genomics* 2008; (9):80.
2. Al-Azawy, A. F. K.. Humodi, A. S. "Phenotypic detection and molecular characterization of extended spectrum β-lactamases in Enterobacteriaceae isolated from Khartoum hospitals." PhD diss. *Sudan Journal of Science and Technology* 2012; ISSN: 1858-6716.
3. Fluit AC, van der Bruggen JT, Aareestrup FM, Verhoesf J; Jansen WT. Priorities for antibiotic resistance surveillance in Europe: *Clinical Microbiology and Infection* 2006; 12: 410-417.
4. Cheesbrough M. *District Laboratory Practice in Tropical Countries*, Part II, Cambridge university press, UK, 2000; chapter 7, pp 36-37-38-167.
5. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests; 21st Informational supplements. *CLSI document* M100-S21, Wayne, Pa: Clinical and Laboratory Standards Institute 2013; 31(1).
6. Willey, J., Sherwood. L, and Woolverton, C. Prescott, Harley & Klein’s Microbiology 7th Edition. McGraw Hill. 106. Murray Patrick. R, Rosenthal. Kens, Kabayashi George S and Pfaallaer. Michael A. (2002). *Medical Microbiology*, 4th edition, Mosby. 2008; P266-279.

7. Eltayeb HN and Hamedelnil, F. Y. Molecular detection of extended spectrum β-lactamases (ESBLs) genes in *E.coli* isolated from urine specimen, Sennar State, Sudan. *International J of Advanced Scientific and Technical Research* 2012; 5(2): ISSN 2249-9954.
8. Eltayeb HN, M. El Amin. Nagwa. Maowia M. Mukhtar. A M Siddig. Mohamed. Molecular characterization and in silico analysis of a Novel mutation in TEM-1 beta-lactamase gene among pathogenic *E. coli* infecting a Sudanese patient. *American Journal of Microbiological Research* 2014; 6(2) 217-223.
9. Dereeper A., Audic S., Claverice J.M., Blanc G. BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evol. Biol* 2010; (PubMed).
10. Dress, M., D, R., Snydman, C. H. Schmid, L. Barefoot, K. Hansjosten, P. M. Vue, M. Cronin, S.A. Nasraway, and Y. Golan. Antibiotic exposure and room contamination among patients colonized with vancomycin-resistant enterococci. *Infect Control Hosp. Epidemiol* 2008; 29: 709-15.
11. Gupta V. An update on newer beta-lactamases, *Indian J Med Res* 2007; 126:417-427.
12. Moyo SJ, Aboud S, Kasubi M, Lyamuya EF, Maselle SY. Antimicrobial resistance among producers and non-producers of extended spectrum beta-lactamases in urinary isolates at a tertiary hospital in Tanzania. *BMC Res Notes* 2010; 3:348.
13. Tschudin-Sutter S, Frei R, Battegay M, Hoesli I, Widmer AF. Extended spectrum β-lactamase-producing *E. coli* in neonatal care unit. *Emerg Infect Dis* 2010; 16(11):1758-1760.
14. Mekki AH, Hassan AN, Elsayed DE. Extended spectrum beta lactamases among multi drug resistant Escherichia coli and Klebsiella species causing urinary tract infections in Khartoum. *Journal of Bacteriology Research* 2010; 2 (3): 18-21.
15. Mohamed Al-Agamy MH, *et al.* First description of CTX-M beta-lactamase-producing clinical *E. coli* isolates from Egypt. *Int J Antimicrob Agents* 2006; 27 (6) : 545-548.
16. Delela G, Gupta ,S, Jain DK, Mehta P. Antibiotic Resistance Pattern in Uropathologensis at a tertiary care hospital at Jhalawar with special reference to ESBL, Ampc b-lactamases and MRSA production. *J Clin DR* 2012; 6(4): 645-651.
17. Noyal MJ, Menezes GA, Harish BN, Sujatha S, Parija SC. Simple screening tests for the detection of carbapenemases in clinical isolates of non-fermentative, Gram-negative bacteria. *Indian J Med Res* 2009; 129:707-12.
18. Rudresh SM, Nagarathnamma T. Extended spectrum β-lactamase producing *Enterobacteriaceae* & antibiotic co-resistance. *Indian J Med Res* 2011; 133:116-118.
19. Ahmed AA, Osman H, Mansour AM, Musa HA, Ahmed AB, Karrar Z. Antimicrobial agents' resistance in bacterial isolates from patients with diarrhea and urinary tract infection in the Sudan. *The American Journal of Tropical Medicine and Hygiene* 2000; 63: 259-263.
20. Quinteros, M, Radice M, Gardella N, Rodriguez M. M, Costa N, Korbenfeld D, Couto E, and Gutkind G. Extended spectrum beta-lactamases in *Enterobacteriaceae* in Buenos Aires, Argentina, public hospitals. *Antimicrob Agents Chemother* 2003; 47: 2864-2867.
21. Sekar B, Shwetha R, Arunagiri K, Menaka K, Lalitha P, Aparna V. Detection and characterization of bla CTX-M gene by PCR-RFLP analysis among third generation cephalosporin resistant Gram negative isolates. XXX National Congress of Indian Association of

Medical Microbiologists. *Microconi* 2006; P.27.
22. Tasli H, Bahar IH. Molecular Characterization of TEM and SHV derived ESBL in hospital based Enterobacteriaceae in Turkey. *Jpn. J. Infect, Dis* 2005; 58: 162-167.