Detection of CTX-M, TEM and SHV Genes in Gram Negative Bacteria Isolated from Nosocomial Patients at Port Sudan Teaching Hospital

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Abstract: The presence of ESBLs in many Gram negative *strains* are of serious concern, since these organisms are the most common cause of different human infections. 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. The presence of CTX-M, TEM and SHV genes was confirmed in 6588/ (73.9%) of the isolates. The ESBL genes were detected in 47 *Escherichia coli*, 14 *Klebsiellapneumoniae*, 2 *Proteus mirabilis*, 1 *Serratiaodotifera* and 1 *Enterobactersakasaki*. The nucleotide sequences were subjected to BLAST for sequences similarity and homology.

Keywords: E. coli, K. Pneumoniae, ESBLs, CTX-M Genes, TEM Gene, SHV Genes, Sudan

1. Introduction

ESBL enzymes are plasmid borne and they have evolved from point mutations which altered the configuration of the active site of the original and long known β-lactamases, which have been designated as (TEM-1, TEM-2, and SHV-1). Although most of the ESBLs are mutants of the TEM and the SHV enzymes, the CTX-M type beta lactamases have become more important. The CTX-M type of enzyme constitutes a distinct lineage of the molecular class A β lactamases, which are a rapidly growing group [1].

Since the first description of plasmid-mediated extended spectrum beta lactamase (ESBL) in 1983, The ESBL-producing gram-negative organisms have posed a significant threat to hospitalized patients due to their hydrolyzing activity against extended spectrum cephalosporins often employed in the treatment of hospital-acquired infections. Detection of organisms harboring ESBLs provides clinicians with helpful information. Treatment of infections caused by ESBL-producing organisms with extended-spectrum cephalosporins or aztreonam may result in treatment failure even when the causative organisms appear to be susceptible to these antimicrobial agents by routine susceptibility testing [2]. In addition, patients colonized or infected with ESBL-producing organisms should be placed under contact precautions to avoid hospital transmission. These benefits warrant the detection of ESBL-producing organisms in clinical laboratories [1].

The ESBLs are derivatives of common β-lactamases (TEM and SHV β-lactamases) that have undergone one or more amino acid substitutions near the active site of enzyme, thus increasing their affinity and the hydrolytic activity against third generation cephalosporins and other β-lactam antibiotic. Extensive use of newer generation cephalosporins has been the strong factor for the evolution of newer B-lactamases such as ESBLs. The later are encoded by transferable conjugative plasmids, which often code resistance determinants to other antimicrobial agents such as aminoglycosides. These conjugative plasmids are responsible for the dissemination of resistance to other members of Gram negative bacteria in hospitals and in the community [3].

The development of antimicrobials resistance can be viewed as a global problem in microbial genetic ecology. It is a very complex problem to study, let alone solve, due to the geographic scale, the variety of environmental factors and the vast number

and diversity of microbial agents. The ESBLs continue to be a major problem in clinical setups worldwide, conferring resistance against extended spectrum cephalosporins [4].

ESBL producing bacteria may not be detectable by routine disk diffusion susceptibility test, leading to inappropriate use of antibiotics and treatment failure [5].

The presence of an ESBLs-producing organism in a clinical infection can cause significant treatment problems because ESBL-mediated resistance may result in treatment failure if any of the third generation cephalosporins (e.g. ceftazidime, cefotaxime, and cefttriaxone) or a monobactum (aztreonam) are used [6]. ESBL-producing organisms may also be difficult to detect because of the effect of their different levels of activity against various cephalosporins, thus making the choice of which cephalosporin to test critical. If an ESBL-producer is detected, it should always be reported as resistant to the penicillins, cephalosporins, and monobactams even if in vitro test results indicate susceptibility, since these may fail in treatment [7].

The ESBL producers are becoming more complex and diverse. This will create challenges for those involved in detection of ESBLs in clinical microbiology. However, many clinical laboratories are facing problems in detecting extended-spectrum beta-lactamases (ESBLs). Failure to detect these strains may contribute to uncontrolled spread of nosocomial infections and sometime therapeutic failures. Clinical laboratories need to have adequate facilities to provide a clinically relevant antibiotic testing in hospitals where antibiotic resistance is encountered. Nosocomial antibiotic susceptibility may become necessary and need to be carefully considered in combination with clinical data [8].

2. Materials & Methods

2.1. Bacterial Isolates

Four hundred bacterial isolates were obtained from various clinical specimens including Urine, Blood, Wound swab, Ear swab and Miscellaneous body fluids were collected from infected patients at Port Sudan Teaching hospital. The microbiology laboratory proceeds the specimens for the isolation and identification of significant bacterial pathogens following standard conventional procedures [8, 10].

Specimens of urine and miscellaneous body fluids were collected from the patients into sterile plastic containers and were transported to the microbiology laboratory and they were processed immediately for detection of pathogenic Gram-negativebacteria. However, the blood samples were extracted under aseptic condition and transferred immediately to sterile bottles containing brain heart infusion broth. Specimens from ear and wounds were taken by swabs, then placed on transport media and were analyzed as soon as possible.

2.2. Isolation and Identification of Gram-negative Bacilli

Isolation and identification of gram-negative bacilliwere carried out in a systemic way according to standard microbiological methods [9, 10]. A general procedure for

isolated bacteria included isolation, identification, antimicrobial susceptibility testing and screening to presence of nosocomial isolates expressing an extended-spectrum beta-lactamaseas (ESBLs) by detection of reduced zone of inhibition around the third generation cephalosporins disc as recommended by the Clinical and Laboratory Standards Institute (CLSI). These isolates were confirmed for phenotypic ESBL production by the double disc synergy test (DDST) and the confirmatory double disc diffusion test (DDDT) and then were further confirmed by genotypic method (PCR) [11].

2.3. Cultivation of Specimens

Isolation of Gram-negative bacteria from specimens of urine was done by culturing directly onto CLED, MacConkey and Blood agar plates (Oxoid, Basingstoke England), using sterile nichrome wire calibrated loop. While the isolation of clinical specimens of body fluids was done by culturing directly onto MacConkey and Blood agar plates. The isolation of Gram-negative bacteria from clinical specimens of the ears and wound swabs was done by inoculating directly onto MacConkey agar plates by streaking the swabs onto a small area of the plate. Then the sterile loop was used for cross-streaking to spread the inoculum over the surface of the plate to obtain single colonies. Specimen of blood was received in the microbiology laboratory in a 25 ml brain-heart infusion broth. The bottles were then incubated aerobically overnight at a temperature of 37°C. After overnight incubation, the blood cultures were then subcultured on blood and Macconkey agar plates (first subculture). The plates were then incubated overnight under aerobic conditions. On the third day, the first subcultures were observed for growth, and any growth identified. The samples that did not record any growth were re-incubated for another 24 hours under the same conditions. Up to three subcultures were performed similar to the procedure mentioned above if there was no growth from previous subcultures [10].

All cultured plates were incubated aerobically for 24 hours at 37°C and were examined for countable colonies. Each single significant growth of Gram-negative bacteria isolates were identified on the basis of cultural characteristics, gram stains, oxidase test and conventional biochemical tests, then confirmed by API 20E identification system (biomerieux Marcy-I'Etoile, France). Culture plates which yielded more than two organisms per specimen were excluded from the study.

2.4. Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing of Gram negative bacteria isolates was performed on Mueller-Hinton agar plate (Oxoid, Basingstoke England) by the Kirby-Bauer disk diffusion method following the CLSI recommendations. Allisolates were tested for their susceptibility against 14 antimicrobial agents including; amikacin (30 μg), amoxicillin (10 μg), amoxicillin-clavulanic acid (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin

(10 μg), nalidixicacid (30 μg), nitrofurantoin (50 μg), tetracycline (30 μg), tobramicin (10 μg) and trimethoprim-sulfamethoxazole (25 μg), (Liofilchem Co. Italy). Standardized inoculum conforming to 0.5 McFarland standard turbidity of each isolate was inoculated on two Mueller-Hinton agar plates using a sterile cotton swab by streaking the swab over the entire sterile agar surface 3 times. Then onto each plate, 8 to 9 antimicrobial disks were placed at the recommended distance from each other. All plates were aerobically incubated at 37°C for 18 hours before the zone sizes were recorded. *E. coli* ATCC 25922, which were obtained from the American Type Culture Collection was used as control strains and tested each time when susceptibility testing was performed. Test results were only validated in the cases where inhibition zone diameters of the control strains were within performance range in accordance to CLSI guidelines [11].

2.5. Phenotypic Detection of ESBLs

2.5.1. ESBL Screening Tests

Screening test was carried out simultaneously with antibiotics sensitivity tests. Screening test was carried out on Muller Hinton agar plates which were seeded by bacterial suspension as mentioned previously. Antibiotics discs, cefotaxime (CTX) 30μg, ceftazidime (CAZ) 30μg, aztreonam (ATM) 30μg, cefpodoxime (PX) 10μg and ceftriaxone (CRO) 30μg, (Liofilchem Co. Italy), were placed aseptically on the plates and pressed gently to the agar surface using sterile forceps then incubated at 35—37°C for 16-18 hours and examined for the inhibition zones. The size of the inhibition zones was compared with zone diameter recommended by CLSI screening criteria, as followed cefotaxime (CTX) screening breakpoint ≤ 27 mm, ceftazidime (CAZ) ≤ 22 mm, aztreonam (ATM)≤ 27 mm, cefpodoxime (PX) ≤ 22 mm and ceftriaxone (CRO) ≤ 25 mm), were considered as potential ESBL producer [11].

2.5.2. Double Disc Synergy Test (DDST)

The double disc synergy test was carried out on Muller-Hinton agar plate seeded by bacterial suspension. A disc containing the amoxiclave (amoxicillin 20 μg plus clavulanic acid 10 μg) was placed on the center of Muller-Hinton agar, four discs of the following cephalosporins; cefepeme 30μg, ceftazidime 30μg, cefotaxime 30 μg, and aztreonam 30 μg were placed around amoxiclave (Augmentin 20/10μg) at distance 25mm center to center. After overnight incubation, if there is an extension of the zone towards the disc containing amoxicillin-clavulanic acid it indicated that the strain possesses an ESBL [11].

2.5.3. Double Disk Diffusion Test (DDDT)

According to NCCLS, and CLSI-ESBL, phenotypic confirmatory test with Ceftazidime 30 μg, cefotaxime 30 μg, and cefepeme 30 μg were performed for all the isolates by disk diffusion test (DDDT). Each disk was placed onMuller-Hinton agar plates with and without 10μg of clavulanic acid. A difference of ≥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 [11]. *E. coli* strain ATCC 25922 was used as a negative control and *Klebsiellapneumoniae* ATCC 700603 was used as a positive control.

2.6. Genotypic Detection of ESBLs

2.6.1. DNA Extraction

The plasmid DNA was extracted using DNA extraction kits (iNtRON BIOTECHNLOGY, Seongnam, Korea), which was shown high quality and quantity of DNA collected from cultured gram-negative bacteria.

All isolates were screened for the resistance genes *ble*CTX-M, *ble*TEM, *and ble*SHV by polymerase chain reaction (PCR), with specific primers. The amplification was done using CONVGYS® td peltier thermal cycle (Gmbh and Co. KG, Germany). In a total reaction volume of 25 ulcontaining (5ul master mix of Maxime RT premix kit (iNtRON BIOTECHNOLOGY, Seongnam, Korea), 0.6 forward primer, 0.6 reverse primer, 2ul plasmid DNA and 16.8ul deionized sterile water. Then thePCR mixture was subjected to initial denaturation step at 94°C for 5-mint, followed by 30 cycles of denaturation at 94°C for 45 seconds, primer annealing at 57°C for 45 seconds, followed by step of elongation at 72°C for 60 seconds, thefinal elongation at 72°C for 5mint. [12].

2.6.2. DNA Sequencing for Characterization of CTX-M, TEM and SHV Genes

Sequencing is a method for determining the nucleotide sequence of a DNA molecule, thus it is a very precise method. DNA purification and standard sequencing was performed for both strands of CTX-M, TEM and SHV genesby Macrogen Company (Seoul, Korea). Selected positive sample were sealed in sterile eppendorf tubes and sent to the DNA sequencing service in Seoul, Korea [13].

2.6.3. Bioinformatics Analysis

The sequences chromatogram was viewed by Finch TV program, (http://www.geospiza.com/Products/finchtv.shtml). Then the nucleotides sequences of the CTX-M, TEM and SHV beta-lactamases genes were searched for sequences similarity using nucleotide BLAST (http: //blast.ncbi.nlm.nih.gov/ Blast.cgi). Highly similar sequences were retrieved from NCBI and subjected to multiple sequence alignment using (http://www.phylogen.fr/simple.phylogeny.cgi) software [14].
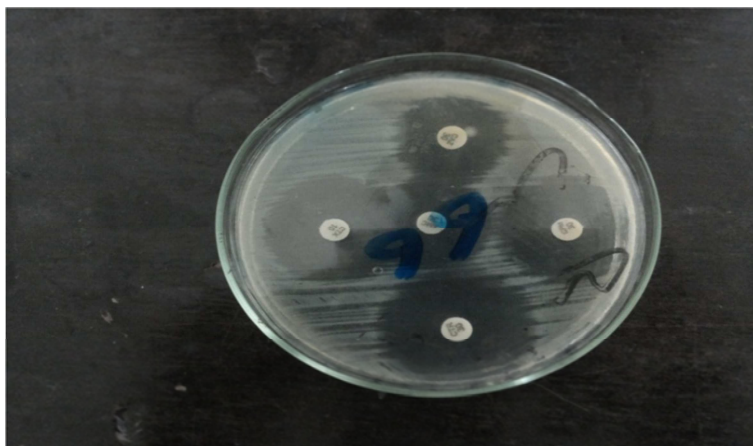
3. Results

3.1. Antibiotic Susceptibility Pattern of Gram Negative Bacilli

Among the ESBL-producing Gram-negative bacilli, high resistance rates were observed for ceftriaxone (100.0%), tetracycline (100.0%), ciprofloxacin (100.0%), amoxyclav (98.9%), cefuroxime (98.9%), nalidixic acid (98.9%) andamoxicillin (95.5%). The highest antimicrobial activities

of ESBL-producing organisms were observed with amikacin (96.6%), followed by Chloramphenicol (63.6%), Tobramicin (53.4%) and nitrofurantoin (50.0%). ESBL-producing Gram-negative bacilli isolates were significantly more resistant to trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid, cefuroxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, nitrofurantoin, amoxyclav, tobramicin and chloramphenicol compared to non-ESBL producing isolates.

3.2. Phenotypic Detection of ESBLs

A total of 198 Gram-negative bacilli isolates were tested for ESBL production. Of the 198 isolates, 94 (47.5%) were found to be ESBL-producers by double-disk synergy test (DDST), and 88 (44.4%) were detected using confirmatory double-disk diffusion test method (DDDT).

Figure 1. *Double-disk synergy test (DDST), showing a clearextension ofthe edge of the inhibition zone of cephalosporin towards amoxicillin-clavulanic acid disk (augmentin) is interpreted as positive for ESBL production.*

Figure 2. *Double-disk diffusion test (DDDT), for each test discs containing cephalosporin alone (cefotaxime 30μg, ceftazidime 30μg, cefepime 30μg) and in combination with clavulanic acid 10μg are applied. The inhibition zone around the cephalosporin disc combined with clavulanic acid is compared with the zone around the disc with the cephalosporin alone. The test is positive if the inhibition zone diameter is ≥ 5 mm larger with clavulanic acid than without.*

3.3. Genotypic Detection of Extended-Spectrum β-lactamases (ESBL) Production

Out of 88 isolates were subjected to PCR. Thegenotypic result (PCR result) confirmed the existence of. 73.9% (65/88) by PCR method, while 26.1% (23/88) was confirmed negative by PCR method.
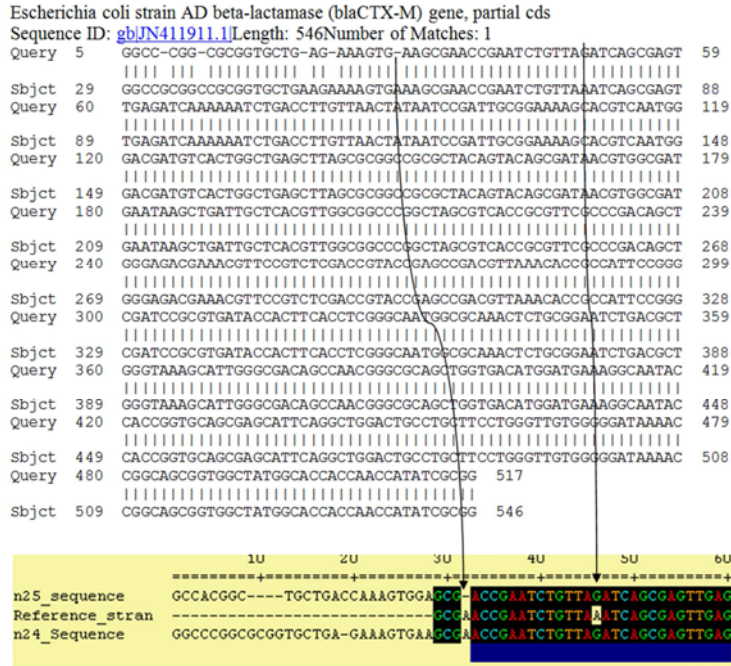
The commonest prevalence of ESBL genes was CTX-M gene 53/88 (60.2%). This was observed in 25/53 isolates (47.2%) of *Escherichia coli*, 6/53 isolates (11.3%) of *Klebsiellapneumoniae* and 1/53 isolate (1.9%) of *Proteus mirabilis*. The difference in the prevalence of ESBL genes was significant. The following gene was TEM gene 22/88 (25.0%), and the least one was the SHV gene 13/88 (14.8%).

Some isolates harbored two or more ESBL genes. Out of the 47 *Escherichia coli* isolates, 2 strains (4.3%) harbored all CTX-M, TEM and SHV genes. While out of 14 *Klebsiellapneumoniae* isolates, 1 strain (7.1%) harbored on CTX-M and SHV genes.
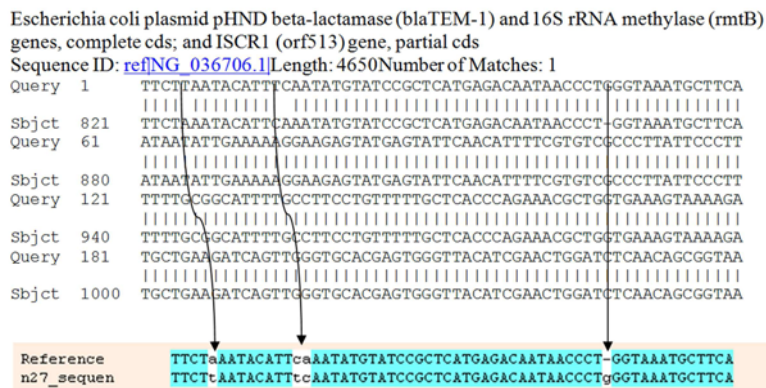
3.4. Multiple Sequence Alignment

The multiple sequence alignment of the mutant isolate with similar nucleotide sequences that obtained from BLASTn was carried out to find the homology and evolutionary relation between these sequences. As shown by BioEdit software there is an inserted and deleted of amino acid at very conserved region, see Figure: (3, 4, 5).
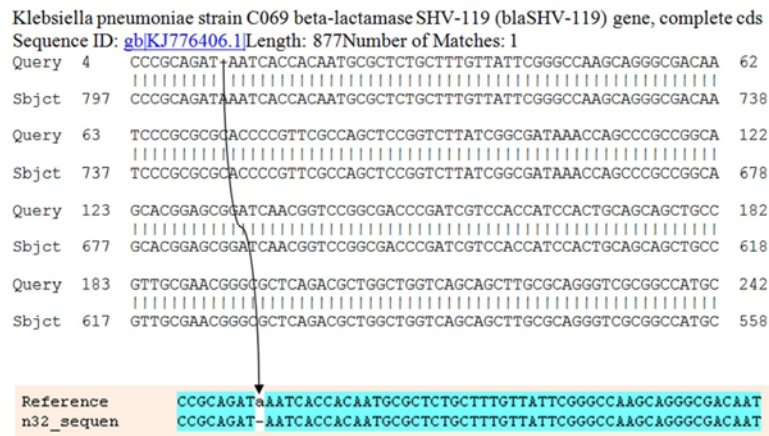
Figure 3. *Sequencing alignment for Mutant CTX-M gene sequence chromatogram. CTX-M gene NO. 25 showed deletion of amino acid adenine at position 31, and substitution of amino acid adenineto guanine (G—A) at position 49, compared to the reference sequence. While CTX-M gene NO. 24 showed a substitution of amino acid adenine to guanine (G—A) at position 49 compared to the reference sequence.*

Figure 4. *Sequencing alignment for Mutant TEM gene sequence chromatogram. 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. And deletion of amino acid guanine at position 47 compared to the reference sequence.*

Figure 5. *Sequencing alignment for Mutant SHV gene sequence chromatogram. SHV gene NO. 32 showed deletion ofamino acid adenine at position 9 compared to the reference sequence.*

4. Discussions

ESBL detection is not routinely carried out in many microbiology laboratories of hospitals in developing countries, 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 [15].

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. Thereforethe Clinical and Laboratory Standards Institute (CLSI) are recommended the use of DDDT for the phenotypic confirmation of the ESBL producers among Gram-negative bacilli. But these both tests were yielded an equal accuracy in the determination of ESBL production. These methods had been previously documented as effective tests for detection of ESBL-producers by other authors. Moreover, these both tests are available and simple to apply routinely along with antimicrobial susceptibility test in our hospital [16].

In this study the EBSL phenotypes were detected 44.4% isolate and confirmed by DDDT method. Non EBSL phenotypes were detected among 55.6% of the isolates studied. This figure is nearly to that figure reported in Sudan where ESBL producerswere 40% [17]., 2012). While this figure is low compared to the figure reported in a study carried out in Khartoum State hospitals where ESBL productions among Gram-negative isolates were 53% [18]. Also lower than the 60.9% observedin Egypt [19]. And 61.6% reported in India [20]. But, the current study findings are similar to that obtained in Sudan where Gram-negative isolateswere 45% [21]. But, much higher than the 6.5% reported in Saudi Arabia [22]. In addition, the observed prevalence of 44.4% in the current study is much higher compared to those reported in Europe, USA and Canada [23]. Overall, these findings indicate that the prevalence of bacteria producing-ESBL varies worldwide.

The present study determined high resistance rates among ESBL-producing strains to first line antimicrobial therapy such as amoxicillin, trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid ciprofloxacin and amoxicillin-clavulanic acid. Similar rates of resistance have been previously reported in Sudan [24]. Other developing countries [25]. Significantly high rates of resistance to such commonly used oral antimicrobials have been previously described making these agents clinically ineffective for empirical treatment of infection caused by ESBL-producing strains [26].

Whilst the cephalosporins such as, cefuroxime, ceftriaxone and ceftazidime have been used to treat Gram-negative bacterial infections of various body sites [27]. In this study, higher resistance rates were observed among isolated strains for ceftriaxone (100%) (p 0.00) and cefuroxime (98.9%) (p 0.002). A similar study inSaudi Arabia conducted ESBL-producing strains were found to show high resistance to ceftriaxone [28]. The high percentage of resistance to third generation cephalosporins notably to ceftriaxone is of great concern, since it was found to be much higher than those reported in other parts of the world [28]. A possible explanation for the high resistance might be due to un-appropriate use of these drugs, or the presence of extended spectrum β-lactamases enzymes (ESBL). Since, ESBL mediated resistant to *β*-lactam antimicrobials of penicillin and cephalosporins groups as well as other classes of antimicrobial agents, it is therefore important that routine screening of ESBL in clinical isolates is carried out to prevent widespread of resistant isolates in our hospital.

In the present study genotypic screening of 88/198 (44.4%) confirmed ESBL phenotype strains by P.C.R had revealed 65/88 (73.9%) frequency rate positive genotypes for at least one of studied genes. While 26.1% of positive phenotype ESBL strains 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. More genotypes were found among *E. coli* 47/65 (72.3%) and *K. pneumonia* 14/65 (21.5%) in a similar finding reported by Moosavian and Deiham., (2012) [30]. found more positive genotypic strains in *E. coli* and *K. pneumoniae* (79.5%). Out of 65 (73.9%) isolated genes. CTX-M gene was occurred at highest frequency 53/88 (60.2%). This result is in-agreement with that reported by Quinteros*et al*., (2003) [31]. However, it has been reported that the proportion of CTX-M strains among ESBL-producing isolates had dramatically increased from 38.2% to 87% worldwide [31]. Less CTX-M prevalence was reported in study carried out in Tehran republic of Iran [32]., and Korea [33]. Also in this study showed that CTX-M gene was most common encountered in *E. coli* with an account of 25/53 (47.2%). This is in-agreement with the reports by Sekar*et al* [34]. in India, who detected that 44.4% was *E. coli*. Butthis figure is low compared to the figure reported by Eltayeb HN *et al* [35]. in Sudan, who recorded CTX-M gene among *E. coli* was (65.2%). In addition, the observed prevalence of CTX-M gene (47.2%) among *E. coli* in the current study is much lower compared to that reported by Kola, *et al* [36]., who found to be (70%). The spread of CTX-M had also been described through prospective studies in industrialized countries such as Canada, France and United Kingdom [37]. The TEM was a second ESBL gene among isolates (25.0%). This is similar to that reported by Al-azawy*et al* [7]. in Sudan, who found to be (22.2%). But less than that (72.0%) reported by Hosoglu *et al*. [38] In our study the SHV was less frequent gene (14.8%). Also this result is in-agreement to that (16.7%) reported by Al-azawy*et al*., (2012) [7]. in Sudan.

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 HN *et al* [7], who reported that 6.5% was SHV gene among *E. coli* strains.

In our study the BLASTn searchingand phylogeny fr.multiple sequence alignment revealedan insertion, deletion and substitution of amino acids to the different genes of CTX-M, TEM and SHV, at very conserved region. This mutation may be the cause of ESBL activity, because this mutation 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 discussed by Eltayeb HN *et al* [39].

5. Conclusions

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. ESBLs genes are rapidly evolved among pathogenic bacteria, thus study like this to detect a new antibiotic resistant gene variants could guide the choice of optimal antibiotic therapy for successful treatment, thus improving the outcomes for patients with severe bacterial infections.

Acknowledgements

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