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Original Research Article

Detection of Nosocomial Isolates Expressing an Extended Spectrum β-lactamase Phenotype in Port Sudan Teaching Hospitals, Red Sea State, Sudan

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Abstract: This study aimed to determine the frequency of extended spectrum beta lactamase producing strains among gram negative bacilli causing nosocomial infections. In addition to determine the drug resistance pattern of using pathogens isolated from nosocomial patients using Kirby-Bauer disc diffusion method. 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. Collected data were analyzed by using Statistical Package for Social Sciences (SPSS; Version 20). P value <0.05 were considered statistically significant. Accordingly, our 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%).
Keywords: E. coli, K. pneumoniae, ESBLs, CTX-M genes, TEM gene, SHV genes, Sudan

INTRODUCTION

The ever-increasing bacterial resistance to antibiotics is one of the most challenging tasks of all the medical issues which are being faced by us today. A single mutation in bacteria which leads to a new resistance mechanism against various drugs is like undoing within moments, the great efforts in developing these drugs, of a great mind. The persistent exposure of the bacterial strains to a multitude of β-lactams has induced a dynamic and continuous production and mutation of β-lactamases in the bacteria, expanding their activity even against the third generation cephalosporins such as ceftazidime, cefotaxime and cefepime and also against aztreonam. These new β-lactamases are called extended spectrum β-lactamases (ESBLs) [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 [3]. ESBL production has become more and more common in a variety of enteric bacilli other than *Klebsiella* spp. or *Escherichia coli* (e.g. *Enterobacter aerogenes*, *E.*

cloacae, *Serratia marcescens*, *Morganella morganii*, *Providentia* spp., *Citrobacter freundii* and *C. koserii*), as well as in non-enteric bacilli (e.g. *Pseudomonas aeruginosa*), also they can now be found in many other species [3]. The introduction of new β-lactam drugs in the treatment of patients has always been met by the emergence of new β-lactamases that caused resistance to the new class. Oxymino-cephalosporins specifically ceftazidime, which was widely used in the 1980's for the treatment of serious Gram negative infections, soon become ineffective as a results of the emergence of ESBL-producers [2]. Extended spectrum beta lactamases (ESBL) hydrolyze expanded spectrum cephalosporins like ceftazidime, cephotaxime which are used in the treatment of nosocomial infections [4]. These enzymes responsible for resistance of Gram negative bacteria to β-lactamase antibiotics like penicillins, cephalosporins, monobactams, and carbapenems. These groups of antibiotics are typically used to treat both of Gram-positive and Gram-negative bacteria. The Beta lactamase enzymes breaks β-lactam ring, deactivating the molecule's antibacterial properties [5].

METHODS

The study was a descriptive cross-sectional study conducted between September 2011 to September 2014. 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 precedes the specimens for the isolation and identification of significant bacterial pathogens following standard conventional procedures [6]. 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-negative bacteria. 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.

Isolation and identification of gram-negative bacilli were carried out in a systemic way according to standard microbiological methods [6]. 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).

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 ^{0}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 [6].

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 [7]. 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. All isolates 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 37ºC 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 [8].

ESBL detection was done on all isolates which were screened for ESBL production by the DDST, then confirmed by the double-disk diffusion test (DDDT) as recommended by the CLSI. 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 oC for16-18 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 [8].

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 [8].

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 on Muller-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 [8]. *E.coli* strain ATCC 25922 was used as a negative control and *Klebsiella pneumoniae* ATCC 700603 was used as a positive control.

RESULTS

Out of 400 samples 298 (74.5%) gave a significant growth, the Gram-negative bacilli was 198 (66.4%) of it, 70 (23.5%) Gram-positive, 10 (3.4%) yeast cells and 20 (6.7%) was mixed organisms. The total of 198 samples of Gram-negative bacterial isolates were recovered from clinical specimens of urine (n=110), wounds (n=67), ear swabs (n=9), miscellaneous body fluids (n=11) and blood (n=1).

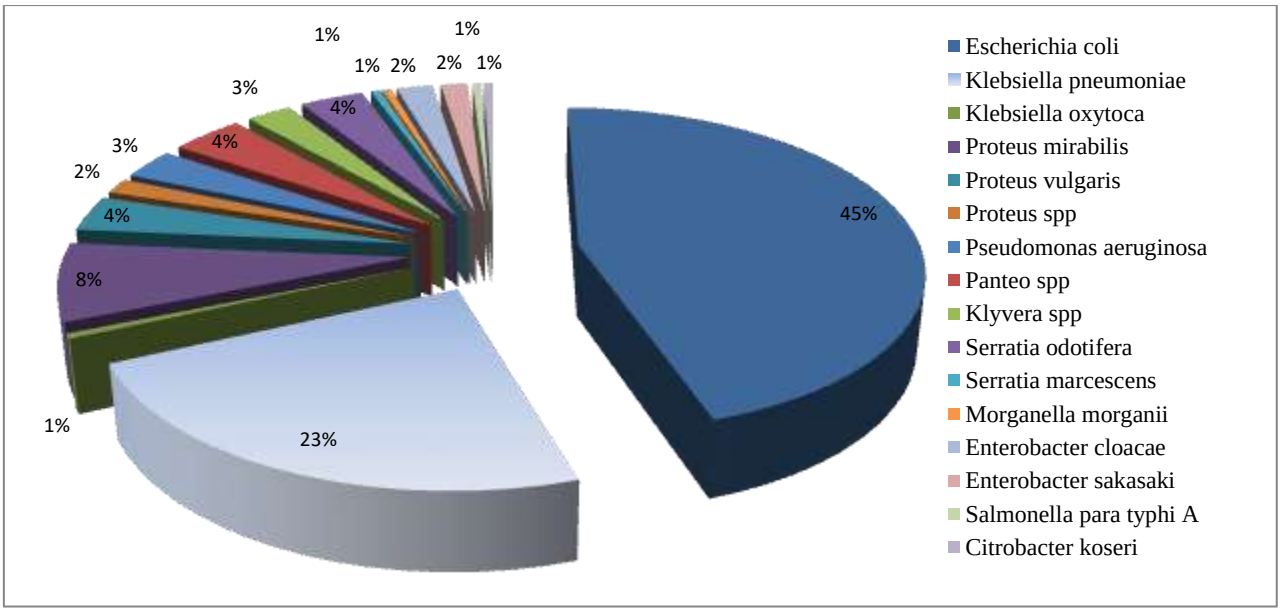

Fig 1: Frequency of isolated organisms

Among the 198 patients infected with Gram-negative bacilli, 125 (63.1%) were females, while 73 (36.9%) were males. This 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, followed by the age group of more than 50 years (94/47.5%), and the lowest frequency of isolates (5/2.5%) in the age group of less than 12 years as shown in fig.3. This age frequency was statistically significant (*p* 0.00).

The results of antimicrobial susceptibility of isolated organisms are shown in Table 6. 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%) and amoxicillin (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 ($p<0.05$) Table 1.

All of the ESBL producing isolates were showed resistance to ceftriaxone, indicating that ceftriaxone was a good drug for the detection of the ESBL activity having significant correlation (p value 0.00). Table 2, 3 summarizes the frequency of ESBL producers among Gram negative bacilli in various clinical specimens and organism wise distribution of ESBL producers.

Table 1: Antimicrobial susceptibility pattern in both ESBL and non ESBL producer

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
Nalidixicacid	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
Tobramicin	53.4	100	46.6	0.0	0.000
Tri.sulfamethoxazole	1.1	22.7	98.9	77.3	0.000

Table 2: ESBL producers among Gram negative bacilli in various clinical specimens

Organism	ESBL producers	
	DDST	DDDT
E.coli	59/94 (62.8%)	56/88 (63.6%)
K.pneumoniae	25/94 (26.6%)	23/88 (26.1%)
P.mirabilis	6/94 (6.3%)	6/88 (6.8%)
Se.odotifera	2/94 (2.1%)	1/88 (2.1%)
Enterobacter cloacae	1/94 (1.1%)	1/88 (1.1%)
E.sakasaki	1/94 (1.1%)	1/88 (1.1%)

Table 3: Specimen and organism wise distribution of ESBL producers

Sample / Organism	Urine	Wound pus	Ear swab	body fluids
	ESBL producer / isolate tested	ESBL producer / isolate tested	ESBL producer / isolate tested	ESBL producer / isolate tested
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%)
En. cloacae	0/110 (0.0%)	1/67 (1.5%)	0/9 (0.0%)	0/11 (0.0%)
En.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%)

DISCUSSION

The present study demonstrated presence of ESBL-producing bacteria 44.4% isolated from clinical specimens of patients in Port Sudan teaching hospital. Several factors contribute to the increased this risk among hospitalized patients, namely the disruption of the normal gastrointestinal flora by administration of broad-spectrum antibiotic, colonization with hospital-associated strains, poor infection control practices, present of indwelling devices including urinary catheters, and an immune suppressed state [9].

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 [10]. 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 [11]. Also it has been observed that these two species were the most prevalence among ESBL-producing microorganisms, confirming international multi-center studies [12, 13]. This is in agreement with our study, where *Escherichia coli* was the most predominant Gram negative isolated 45.5%, followed by *Klebsiella pneumoniae* 23.2%.

ESBL detection is not routinely carried out in many microbiology laboratories of hospitals in developing countries [14], 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 [14]. 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) 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 [15]. Moreover, these both tests are available and simple to apply routinely along with antimicrobial susceptibility test in our hospital.

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 producers were 40% [16]. 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% [17]. Also lower than the 60.9% observed in Egypt [18], and 61.6% reported in India [19]. But, the current study findings are similar to that obtained in Sudan where Gram-negative isolates were 45% [20]. But, much higher than the 6.5% reported in Saudi Arabia [21]. In addition, the observed prevalence of 44.4% in the current study is much higher compared to those reported in Europe, USA and Canada [22]. Overall, these findings indicate that the prevalence of bacteria producing-ESBL varies worldwide. In previous study 65.8%, was ESBL positivity among *E. coli* [23]. These finding correlated well with those of our study, where the occurrence of ESBL producer among *E. coli* was 63.6%. In our study, we also observed that 26.1% *Klebsiella pneumoniae* isolated were ESBL producer. Although *Klebsiella pneumoniae* was more often in Sudan as an ESBL producer, by Ahmed, [24]. Who recorded ESBL production among *Klebsiella pneumoniae* species were 70%. We observed that the ESBL production was more common in the *E. coli* as compared to that in the *Klebsiella pneumoniae* isolate. This result is in-agreement with result reported in India [19].

In *Pseudomonas aeruginosa*, the ESBL production was no detected (0.0%). A possible explanation, because its resistance mechanism was mediated by the production of metallobetalactamase, lack of drug penetration due to mutations in the porins or due to the loss of certain outer membrane proteins and the efflux pump [25]. 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 by Rudresh and Nagarathnamma [23], where 70% of ESBL-producing isolates were obtained from exudates. Recently several factors that may have highly contributed to the occurrence of ESBL-producing isolates in wound infections such as; infection of wounds by microorganisms, which is most often associated with prolonged hospital stay in spite of persistent treatment with antibiotics in different combinations; the attendant risk of acquisition of multiple resistant organisms from medical devices; and hospital environment [26].

In our study, the occurrence of ESBL-producing strains among urine specimens is of great concern 42.7%. *E. coli* is the main causative agent of urinary tract infections represented 35.5%; consequently, there is wide spread use of antimicrobial agents due to such infections. ESBL are becoming an increasing problem for hospitals. The possible explanation for these major risk factors of colonization or infection with ESBL-producing organisms are: long-term antibiotic exposure; prolonged intensive care unit stay; nursing home residency; severe illness; residence in an institution with frequent use of ceftazidime and other third-generation cephalosporin; and instrumentation or catheterization [27]. Therefore, great focus should be directed towards infection control practices in hospital units in order to prevent the spread of ESBL strains from one patient to another through the following means; ensuring healthcare professionals practice hand hygiene, cleaning medical equipment; and preventing colonization of the environment [22]. 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 [28], other developing countries [29]. 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 [30].

Whilst the cephalosporins such as, cefuroxime, ceftriaxone and ceftazidime have been used to treat Gram-negative bacterial infections of various body sites [31]. 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 in Saudi Arabia conducted ESBL-producing strains were found to show high resistance to ceftriaxone [32]. 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 [33, 34]. 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 [35], it is therefore important that routine screening of ESBL in clinical isolates is carried out to prevent widespread of resistant isolates in our hospital.

Like worldwide studies [35], our study was found to be susceptible to aminoglycosides agents notably to (amikacin, tobramicin) and chloramphenicol. Amikacin appears to have wider range of activity than tobramicin; chloramphenicol and others tested antimicrobial agents. Similar result reported in study from India [19], and from Sudan [24], where most Gram negative bacilli were found to be susceptible to Amikacin (67.4%). The explanation for amikacin is probably the fact that these are very powerful drugs used only in hospital setting and not as first-line therapy; therefore, they have lower selective pressure due to their restricted use [31].

In our study, ESBL-producing isolates exhibited significantly higher resistant rates to non-β-lactamase antimicrobials agents including fluoroquinolones aminoglycosides, tetracyclines and trimethoprim-sulfamethoxazole, compared to non-ESBL producing isolates. The possible explanation for this observation may in fact be that ESBLs are encoded on plasmids and can be mobile and therefore, easily transmissible as resistance gene elements for other antimicrobials from one organism to another [18]. In this study female patients were more resistant than those from male patients. This is disagreement with studies conducted recently in Spain [31], and in the USA [36], in which the rates of resistance were observed to increase in isolates from male patients than those from females. As reported by Riaz *et al.;* [37], the antimicrobial resistance was somewhat affected in gender, but that may depend on the type and site of infection. In the present study, age wise distribution of antimicrobial resistance patterns showed variation in percentage of resistance against different antimicrobials. The rate of resistance against cephalosporin classes was found to be 73% in pediatric patients, 60.9% in adult patients, whereas 81.2% was observed among elder patients. This is in-agreement

with Boyd *et al.;* [36], who have observed that resistance to antimicrobials agents increased with age.

CONCLUSION

The occurrence of extended-spectrum *β*-lactamases (ESBL) producing Gram-negative isolates at Port Sudan teaching hospital setting is a serious threat concern and poses resistant determinates to a wide range of antimicrobial agents. *E.coli* and K.pneumoniae were the major ESBL producing pathogen among isolates of Gram-negative bacteria. The alarming finding observed in this study was resistance to third generation cephalosporins. Maximum resistance was detected against ceftriaxone; this is of great concern, which requires sound infection control measures including antimicrobial management to avoid the risk of therapeutic failure and routine laboratory detection of ESBL-producing isolates in order to decrease their spreading.

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