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Research Article

Extended Spectrum Beta Lactamase Producing Bacteria among Critically Ill Patients

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Abstract

Aim: This work aimed at studying the occurrence of extended-spectrum beta-lactamase (ESBL) producing bacteria among critically ill patients. **Subjects and Methods:** A total of 250 different clinical samples were collected from 200 critically ill patients, who were admitted to Al -Salama hospital intensive care unit (ICU). Samples were examined at the microbiology laboratory at the High Institute of Public Health (HIPH) and were subjected to standardized microbiological procedures for isolation and identification of bacterial and fungal agents. Antimicrobial susceptibility testing was done for all bacterial isolates by single disc diffusion method. ESBL producers were detected according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Conventional Polymerase Chain Reaction (PCR) was done to detect Temonieria (TEM) and sulfhydryl variable (SHV) ESBL genes. **Findings:** Causative agents were isolated and identified in 230 samples (92.0%). Of the 236 isolated Gram negative bacilli, 20 isolates were confirmed as ESBL producers (8.5%). Seven ESBL producers (35%) were positive for TEM and SHV genes, distributed as 6 TEM and only one SHV. **Conclusions:** *Klebsiella* spp. was the most prevalent ESBL producer and TEM was the most commonly detected ESBL gene.

Keywords: Extended-spectrum beta-lactamase (ESBL), Enterobacteriacae, Antimicrobial resistance, ICU, ESBL genes, PCR.

Introduction

Antibiotic resistance remains a serious public health problem with a constant increasing rate worldwide. (Bonnet 2004; Yan et al., 2006) The accelerated emergence of antibiotic resistance among the prevalent pathogens due to excessive use of antibiotics in conjunction with the remarkable genetic plasticity of microorganisms became the most serious threat to the management of infectious diseases. Robert (2009).

About 70 % of the bacteria that cause infections in hospitals are resistant to at least one of the antibiotic agents most commonly used for treatment. Some organisms are resistant to all approved antibiotics and can only be treated with experimental and potentially toxic drugs. An alarming increase in resistance of bacteria that cause community acquired infections has also been documented. Todar (2008**)**

A β-lactamase enzyme capable of hydrolysing extended-spectrum cephalosporins was documented, based on genetic and functional characteristics, in strains of *Klebsiella pneumoniae* (*K. pneumoniae*) from Germany. Similar reports from elsewhere in Europe and the United States (U.S.) quickly followed. Because of their spectrum of activity against oxyimino cephalosporins, these enzymes became known as extended spectrum β-lactamases (ESBLs). (Mark and Fey, 2003) Local surveillance was done through Pan European Antimicrobial Resistance [PEARLS] in (2001 - 2002). It showed that the percentages of ESBL production among *Escherichia coli* (*E. coli*), *K. pneumoniae* and *Enterobacter* spp. were 5.4%, 18.2 % and 8.8%, respectively. The overall ESBL production rate for the combined Enterobacteriaceae was 10.5%.

It was highest in Egypt (38.5%) and Greece (27.4%) and lowest in the Netherlands (2.0%) and Germany (2.6%). (Bouchillon 2004; El-Khizzi and Bakheshwain, 2006) In Asia the percentage varies from 4.8% in Korea to 12% in Hong Kong. (Rao et al., 2014)

ESBLs are enzymes capable of hydrolyzing penicillins, broad-spectrum cephalosporins and monobactams, and are generally derived from Temonieria [TEM] and sulfhydryl variable [SHV] type enzymes. ESBL producing organisms pose unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery scientists. (Mark and Fey, 2003)

The high frequency of multi drug resistant organisms (MDROs) in intensive care units (ICUs) necessitate that broad spectrum antibiotics should be prescribed more wisely in order to reduce pressure on sensitive strains. This could be beneficial for saving ICU patients and preventing the spread of resistant isolates in these critical wards. (Bradford, 2001; Bantar et al., 2007; Joint commission.org, 2014)

Recommendations call for routine screening for MDROs in patients admitted to ICUs, with concomitant implementation of additional contact precautions to prevent the diffusion of multi -resistant strains among debilitated patients. (Filippa et al., 2013)

This piece of work aimed to study the occurrence of ESBL producing bacteria among critically ill patients.

Subjects & Methods

The present cross sectional study was conducted through a 6- month period from January 2011 to June 2011.

Sample size:

Assuming the precision of 5% and expected prevalence of 14.6% (10), using confidence level 95% and a risk of 5%, the minimum required sample size = 195.

Samples collection and processing:

This study included 200 critically ill patients, who were admitted to the general ICU of Al-Salama Hospital in Alexandria, with a capacity of 21 beds.

A questionnaire sheet including all the relevant information was filled for every patient. The study was approved by the Ethics Committee at the HIPH.

A total of 250 different clinical samples were collected from the studied patients who had signs and symptoms suggestive of infection, as fever >37oC, chills, bradycardia, redness, discharge and inflamed sites. These samples were collected on admission upon request of the treating physician or whenever needed and were distributed as follows: 95 urine samples, 38 pus and exudate swabs, 35 central venous catheter (CVC) samples, 70 respiratory secretion samples, 10 blood samples, one aspirated fluid sample (knee joint), and one vaginal swab.

All the collected samples were clearly labeled and transported rapidly to the microbiology laboratory at HIPH within 1-2 hours for processing.

At the laboratory, collected samples were subjected to macroscopical and microscopical examinations together with culture on blood, MacConkey's and Sabouraud's agar (SDA) plates.

Identification procedures:

All isolated colonies on blood, MacConkey's and SDA plates were examined morphologically and further identified by microscopic examination and biochemical reactions according to the methods described by Forbes et al. (Forbes et al., 2007)

Antimicrobial susceptibility testing:

All bacterial isolates were tested for their antimicrobial susceptibility patterns using standardized single disc diffusion method described by Bauer and Kirby (Bauer et al., 1966) The test was done on Mueller Hinton agar plates, using the selected antibiotic discs with various concentrations. Inhibition zones were measured, and results were recorded as susceptible (S), intermediate (I), and resistant (R) according to standard tables of Clinical and Laboratory Standards Institute (CLSI). (Patel et al., 2012)

ESBL detection:

All isolates that showed a diameter of less than 27mm for cefotaxime and less than 25mm for ceftriaxone, were selected for checking ESBL production.

Confirmation of ESBL production phenotype was performed by the Double Disc Synergy Test (DDST)

using a disc of amoxicillin-clavulanate (20/10 µg) along with 2 cephalosporin discs: cefotaxime and ceftriaxone. Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for the ESBL production.

Molecular method for identification of DNA probes for SHV and TEM genes by Polymerase chain reaction (PCR).

All the 20 positive ESBL samples were subjected to molecular identification using DNA probes to detect the presence or absence of SHV and TEM using PCR. The DNA was extracted from bacterial isolates, and then amplification was performed in presence of specific primers. The amplified DNA was visualized by the use of gel electrophoresis. (Schmitt et al., 2007)

DNA extraction

Fresh cultures of the tested strains [*K. pneumoniae, E.coli, Proteus* spp.] from blood agar plates were suspended in 500 µl of distilled water and vortexed to get a uniform suspension. The cells were lysed by heating them at 100^{o} C for ten minutes, and cellular debris was removed by centrifugation at 8000 rpm for ten minutes. The supernatant was used as a source of template for amplification. (Schmitt et al., 2007)

I. Amplification Reaction Reagents:

Dream Taq Green PCR Master Mix (2X) (supplied by Fermentas, #K1081)

Dream Taq^{TM} DNA polymerase is supplied in 2x Dream Taq ^{TM} Green buffer, dATP, dCTP, dGTP, 0.4 mM each, and 4 mM $MgCl_2$. Dream Taq^{TM} Green buffer is a proprietary formulation optimized for robust performance in PCR. It contains a density reagent and two dyes for monitoring electrophoresis progress. (Schmitt et al., 2007)

The blue dye migrates with 3-5 kb DNA fragments in a 1% agarose gel and the yellow dye migrates faster than 10 bp DNA fragments in 1% agarose gel. The dyes have absorption peaks at 424 nm and 615 nm. (Schmitt et al., 2007)

Primers (Schmitt et al., 2007)

blaSHV primer.

Forward primer (MN I) (5'-CGC CGG GTT ATT CTT ATT TGT CGC-3')
Reverse primer (MN II) (5'-TCT TTC CGA TGC CGC CGC CAG TCA-3')

blaTEM primer.

TEM Forward primer (5'-ATA AAA TTC TTG AAG ACG AAA-3')
TEM Reverse primer (5'-GAC AGT TAC CAA TGC TTA ATC A-3')

II. Protocol of Amplification (Schmitt et al., 2007)

III. Control: Negative control was prepared by the addition of nuclease free water instead of the extract to the reaction mixture.

The tubes were placed in thermal cycler (Beco, Germany) for amplification according to the following thermal profile.

Table (1): Thermal profile of PCR for SHV (Schmitt et al., 2007)

		Temp.	Time	Cycles
PCR Amplification	Initial denaturation	94^{o}C	1-3 min	1 cycle
	Denaturation	94^{o}C	30 sec	30 cycles
	Annealing	60 ^{o}C	30 sec	
	Extension	72 ^{o}C	60 sec	
	Extension	72 ^{o}C	5 min	1 cycle

Table (2): Thermal profile of PCR for TEM (Alanis, 2005)

		Temp.	Time	Cycles
PCR Amplification	Initial denaturation	94^{o}C	5 min	1 cycle
	Denaturation	94^{o}C	1 min	35cycles
	Annealing	55 ^{o}C	1 min	
	Extension	72 ^{o}C	1 min	
	Extension	72 ^{o}C	5 min	1 cycle

I. DNA detection:

The amplification products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Reagents:

Agarose gel (Promega V312 A)
100 bp blue extended DNA ladder, 0.1 µg/µl (Bioron, Germany, 304105).
Ethidium bromide stain (Promega G188A)
Tris Borate EDTA (TBE 10 X) buffer stock solution
Electrophoresis buffer: Working solution TBE (1X).

Detection procedures: (Schmitt et al., 2007)

1. The gel running plate was placed in its special gel casting plate supplied with electrophoresis apparatus (Mini- plate France) on a horizontal surface to form a mold. The comb was put at 0.5-1.0 mm above the running plate, so the separate wells were formed when the agarose was added.
2. One litre of electrophoresis buffer was prepared to fill the electrophoresis chamber.
3. Two percent (wt/vol) of powdered agarose were added to 100 ml of electrophoresis buffer in a glass flask, and microwaved for 30s until agarose dissolved.
4. The agarose was tempered to 50 – 60°C and 5 µl of ethidium bromide stock solution (1%) were added and mixed by swirling to give a final concentration of 0.5µg/ml.
5. Agarose was poured carefully into gel casting platform fitted with the comb (3-5 mm thick).
6. After solidification of the gel at room temperature (30 min), the comb was carefully removed and the gel on its running plate was mounted in the electrophoresis chamber.
7. TBE buffer was added, just enough to cover the gel to a depth of about one mm.
8. Twenty µL of PCR products for both (SHV and TEM) and DNA ladder were added to wells using micropipettes.
9. The lid of the electrophoresis chamber was closed and the electrical leads were attached so that the DNA will migrate towards anode. The voltage applied was 110 volts for about one hr.
10. The electric current was turned off and the lid was removed once the dye has migrated an appropriate distance (about 10 cm) through the gel.
11. The DNA bands were visualized with UV transilluminator. The gel was examined for specific bands :

For amplification of genes encoding SHV: 1016 bp PCR product that contained the entire opening reading frame (ORF) as determined by molecular weight markers run at the same time was examined.

1 2 3 4 5 6 7 8 9 10

Figure (1): Detection of SHV enzyme by PCR
Lane 1: Marker (100 DNA ladder)
Lane (10) was positive SHV
Lane 21: negative control

For amplification of genes encoding TEM: 1080 bp amplicon reached from 214 bp upstream of the start codon to the stop codon as determined by molecular weight markers run at the same time was examined.

21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Figure (2): Detection of TEM enzyme by PCR
Lane 1: Marker (100 DNA ladder)
Lanes (1, 2, 9, 8, 10, and 20) were positive TEM
Lane 21: negative control

Statistical analysis: (Daniel, 2009)

The results of the present work were tabulated and statistical analysis was carried out by different significance tests according to the situation of comparison.

- X^2 test: used for testing association between two variables.
- MCP: P value based on Mont Carlo exact probability.

Results

The results of the present study showed that causative agents were isolated and identified in 230 samples (92.0%), while in the remaining 20 samples (8.0%) no agents were isolated. Of the 236 isolated Gram negative bacilli, 20 isolates were confirmed as ESBL producers (8.5%).

Of the 200 critically ill examined patients, 105 (52.5%) were of age group less than 60 years. They included 110 (55.0%) males and 90 (45.0%) females, with mean age of 66.7 (±22.9) years ranging between 30 and 85 years old. Only 30 (15.0%) were non Egyptians. The rest were Egyptians distributed as 130 (65.0%) from urban areas and 40 (20.0%) from rural areas. Regarding risk factors, the highest factor among these patients was underlying diseases constituting 46.0%, followed by antimicrobial therapy for the last 3 months (39.8%), and long hospital stay (20.5%). DM was the most frequently associated clinical disease (30.4%). (Table 3)

Table (3): Demographic and clinical characteristics of the 200 studied critically ill patients.

Studied variable		Category	Number	Percent
Age group (years)		Less than 60	105	52.5
		60 or more	95	47.5
Gender		Male	110	55.0
		Female	90	45.0
Residence	Egypt	Urban *	130	65.0
		Rural**	40	20.0
	Other countries ***		30	15.0
Risk factors	Underlying diseases (92)			46.0
	DM		28	30.4
	Renal impairment		20	21.7
	Pneumonia		20	21.7

	Malignancies	24	26.2
	Other risk factors (88)		**44.0**
	Readmission	7	7.9
	Long hospital stay	18	20.5
	Transfer from other facilities	28	31.8
	Antimicrobial intake in the last 3 months	35	39.8
	None	20	10.0

N.B.
*Urban = [Alexandria, Cairo].
** Rural = [Kafr El Dawar, Damanhour].
*** Other countries = [Libya, Saudi Arabia, U.S., Malaysia]

Of the 230 positive patient samples, 59.1% revealed single isolates, while 40.9% were mixed. Single isolates were more frequently encountered than mixed ones in urine, sputum and CVC samples with the following percentages 58.3%, 62.0%, and 66.6%, respectively. On the other hand, mixed isolates were more frequently encountered than single ones in pus and exudate samples (57.9%, 42.1%), respectively. Vaginal swab, aspirated fluid and blood samples yielded single isolates only (100.0%) each. No significant difference was found between these results. (Fig 3)

As shown in table (4), the 324 isolates from 230 positive patient samples were distributed according to the frequency of isolation. Gram negative bacilli were the most frequently isolated agents 236/324 (72.9%), followed by Gram positive cocci 68/324 (20.9%).Fungi were the least among isolated agents representing only 20/324 (6.2 %) of the isolates. It is also evident that of the 236 Gram negative bacilli, *K. pneumoniae* was the most frequently isolated organism representing 85(26.2%), followed by *E.coli* 75 (23.2%), *Pseudomonas aeruginosa* (*P.aeruginosa*) 45 (13.8%), *Acinetobacter baumanii 24* (7.4%) *and Proteus* spp. 7 (2.2 %). As regards the 68 Gram positive cocci, Coagulase negative staphylococci (CoNS) were the most frequently encountered organisms accounting for 14.2 % of the isolates followed by *S.aureus* (5.6%), while regarding fungi, *Candida albicans* (*C.albicans*) represented 6.2% of the isolates.

Figure (3): Pattern of isolation of causative organisms from 230 positive patient samples in relation to clinical samples.

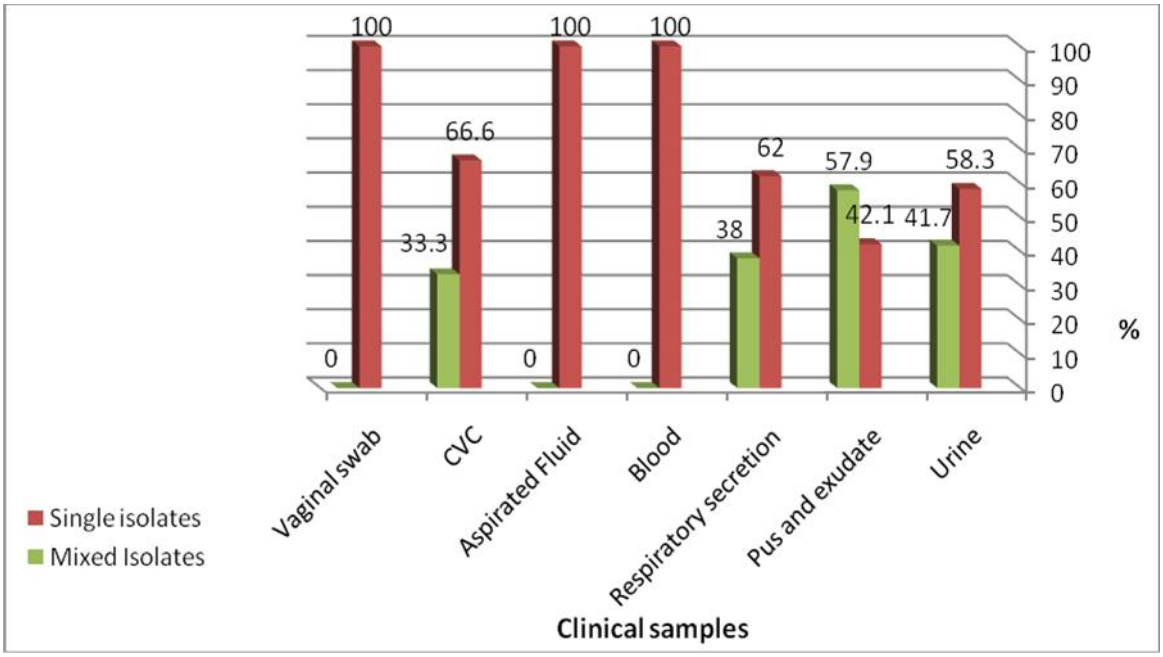

Table (4): Distribution of 324 isolates from the 230 positive patient samples.

Isolated Organisms	Frequency of isolation	
	No.	**%**
Gram positive cocci	**(68)**	**20.9**
S.aureus	18	5.6
MRSA	3	0.9
CoNS	46	14.2
Micrococci	1	0.3
Gram negative bacilli	**(236)**	**72.9**
E.coli	75	23.2
K. pneumoniae	85	26.2
Proteus spp.	7	2.2
P.aeruginosa	45	13.8
Acinetobacter baumanii	24	7.4
Fungi	**(20)**	**6.2**
C. albicans	20	6.2
Total	**324**	**100.0**

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This work showed that oxacillin sensitive CoNS was the most commonly isolated organism in urine, pus and exudate, sputum and CVC samples with the following percentages 33.3%, 66.6%,47.6%,and 55.5%, respectively. For blood samples, *S.aureus* was the only isolated organism. *E.coli* was the most commonly encountered isolate in urine and pus and exudate samples (56.3% and 52.1%, respectively). The remaining samples apparently differed in distribution as *Klebsiella* took the upper hand accounting for 100.0%, 80.0%, 75.0% and 40.7% in aspirated fluid, blood, CVC and sputum samples, respectively.

High sensitivity to amikin and carbapenems was noticed in *Klebsiella*, (100, 0%, 94.1%), respectively, while low sensitivity was observed to ampicillin-sulbactam 54.1%. Regarding cephalosporins, high sensitivity was for cefoperazone and cefipime (92.9%, 90.5%), respectively. Low sensitivity was for quinolones in *P.aeruginosa* with maximum percentage 77.6%, while high sensitivity was noticed in carbapenems 86.6%. Sensitivity to each of cefipime and cefazolin was found in 84.4 % of *P.aeruginosa* isolates in this study. (Table 5)

Of the 12 male patients with ESBL producers, 58.3% were 60 years old or more and 41.6% were less than 60 years. While, the 8 females with ESBL producers were equally distributed among the two age groups, 50.0% each. (Table 6)

Among ESBL producer patients, DM and pneumonia were the most frequent co-morbidity risk factors 25.0% each, followed by malignancies and renal impairment 20.8% and 15.0%. The highest percentage of ESBL producing bacteria was among patients who had indwelling urinary catheters (20.0%), followed by patients with CVC (15.0%). (Table 7)

Table (5): Antimicrobial susceptibility of the 304 bacterial isolates recovered from 230 positive patients' samples.

Isolated bacterial agents / Tested Antibiotics	Gram+ve								Gram -Ve									
	S.aureus (18)		*MRSA (3)*		*CoNS (46)*		*Micrococci (1)*		*E.coli (75)*		*Klebsiella (85)*		*Pseudomonas (45)*		*Proteus (7)*		*Acinetobacter (24)*	
	S		S		S		S		S		S		S		S		S	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Penicillin																		
Ampicillin	ND	ND	ND	ND	ND	ND	ND	ND	67	89.3	70	82.3	ND	ND	3	42.8	9	37.5
Piperacillin	ND	ND	ND	ND	ND	ND	ND	ND	62	82.7	60	70.5	ND	ND	6	85.1	11	45.8
Oxacillin	ND	ND	ND	ND	ND	ND	ND	ND	61	81.3	66	77.6	ND	ND	4	57.1	12	50.0
β- lactam/ β- lactamase inhibitor combinations																		
Amoxicillin - Clavunate	18	100	3	100	2	4.2	0	0	61	81.3	57	67.1	35	77.7	5	71.4	5	20.8
Ampicillin-sulbactam	18	100	3	100	2	4.2	0	0	67	89.3	46	54.1	21	46.6	6	85.7	11	45.8
Piperacillin-tazobactam	0	0	0	0	3	6.3	0	0	67	89.3	45	52.9	22	48.8	4	57.1	9	37.5
Cephalosporins																		
Cefazolin	0	0	0	0	2	4.2	0	0	62	82.7	75	88.8	38	84.4	5	71.4	9	37.5
Cefepime	0	0	0	0	2	4.2	0	0	61	81.3	77	90.5	38	84.4	3	42.8	9	37.5
Cefoperazone	0	0	0	0	2	4.2	0	0	60	80.0	72	84.7	35	77.7	6	85.1	11	45.8
Cefotaxime	0	0	0	0	2	4.2	0	0	60	80.0	72	84.7	34	75.5	4	57.1	12	50.0
Ceftriaxone	0	0	0	0	2	4.2	0	0	62	82.7	75	88.8	32	71.1	5	71.4	12	50.0
Cefoxitin	0	0	0	0	47	97.9	1	100	61	81.3	71	83.5	38	84.4	5	71.4	10	41.6
Cefoperazone + Sulbactam	0	0	0	0	3	6.3	0	0	61	81.3	79	92.9	34	75.5	6	85.1	10	41.6
Aminoglycosides																		
Amikin	18	100	3	100	46	95.8	0	0	75	100	85	100	45	100	7	100	24	100
Gentamicin	0	0	0	0	6	12.5	0	0	65	86.7	70	82.4	31	68.9	7	100	20	83.3
Quinolones																		
Ciprofloxacin	18	100	3	100	45	93.8	1	100	67	89.3	70	82.3	35	77.7	6	85.1	12	50.0
Levofloxacin	0	0	0	0	5	10.4	0	0	66	88.0	60	70.5	32	71.1	4	57.1	19	79.1
Norfloxacin	0	0	0	0	5	10.4	0	0	60	80.0	66	77.6	29	64.4	5	71.4	12	50.0

Carbapenems																		
Imipenem	0	0	0	0	44	91.7	1	100	75	100	80	94.1	39	86.6	7	100	20	83.3
Meropenem	0	0	0	0	44	91.7	1	100	75	100	80	94.1	39	86.6	7	100	20	83.3
Glycopeptides																		
Vancomycin	18	100	3	100	46	100	1	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Linzezolid	18	100	3	100	46	100	1	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Teicoplanin	18	100	3	100	46	100	1	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Lincosamides																		
lincomycin	0	0	0	0	6	12.5	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Clindamycin	3	100	3	100	4	8.3	0	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tetracyclines																		
Doxycycline	0	0	0	0	2	4.2	0	0	65	86.6	60	70.5	ND	ND	5	71.4	12	50.0
Trimethoprim																		
Trimethoprim-sulfamethoxaxole	3	100	0	0	6	12.5	0	0	66	88.0	75	88.2	35	77.7	6	85.7	20	83.3
Nitrofurantoin																		
Nitrofurantoin	3	100	3	100	3	6.3	0	0	70	93.3	77	90.5	39	86.6	6	85.7	20	83.3
Monobactam																		
Aztreonam	0	0	0	0	2	4.2	0	0	38	50.6	71	83.5	34	75.5	5	71.4	11	45.8

ND: Not Done 0 = resistant

Table (6): Distribution of the 20 patients infected with ESBL producers according to their age and sex.

Sex / Age (years)	Males (12)		Females (8)		Total (20)	
	No.	%	No	%	No	%
Less than 60 years	5	41.6	4	50.0	9	45.0
60 years and more	7	58.3	4	50.0	11	55.0
Total	12	100	8	100	20	100
X^2 (P)	**0.14 (0.713)**					

Table (7): Risk factors among the 20 examined patients with ESBL producers.

Risk factors	Patients with ESBL producers (20)		X^2 P value
	No.	%	
Age			
Less than 60 (105)	9	8.5	0.41
60 years and more (95)	11	11.5	0.522
Gender			
Male (110)	12	10.9	0.18
Female (90)	8	8.8	0.66
Related devices			
Mechanical ventilator (MV) (108)	2	1.8	23.15 Less than 0.005*
Central venous catheter (CVC) (67)	3	4.5	43.56 Less than 0.005*
Indwelling urinary catheter (46)	4	8.7	25.76 Less than 0.005*
Readmission (7)	4	57.1	4.88 0.18
Long stay in ICU (18)	4	22.2	
Transfer from other facilities (28)	3	10.7	
Antimicrobial exposure for last 3 month (35)	5	14.2	
Underlying disease			
DM (28)	7	25.0	0.56 0.91
Renal impairment (20)	3	15.0	
Pneumonia (20)	5	25.0	
Malignancies (24)	5	20.8	

P value = * Significant

Among 20 identified ESBL producing bacteria, 7/20 were positive for TEM and SHV genes, distributed as 6 TEM and only one SHV. Of the 6 (30.0%) ESBL *Klebsiella*, 6(85.7%) had TEM gene. On the other hand, ESBL *E.coli* revealed only one (14.3%) SHV gene. (Figure 5)

Figure (5): Distribution of the 20 ESBL producer isolates in relation to clinical samples.

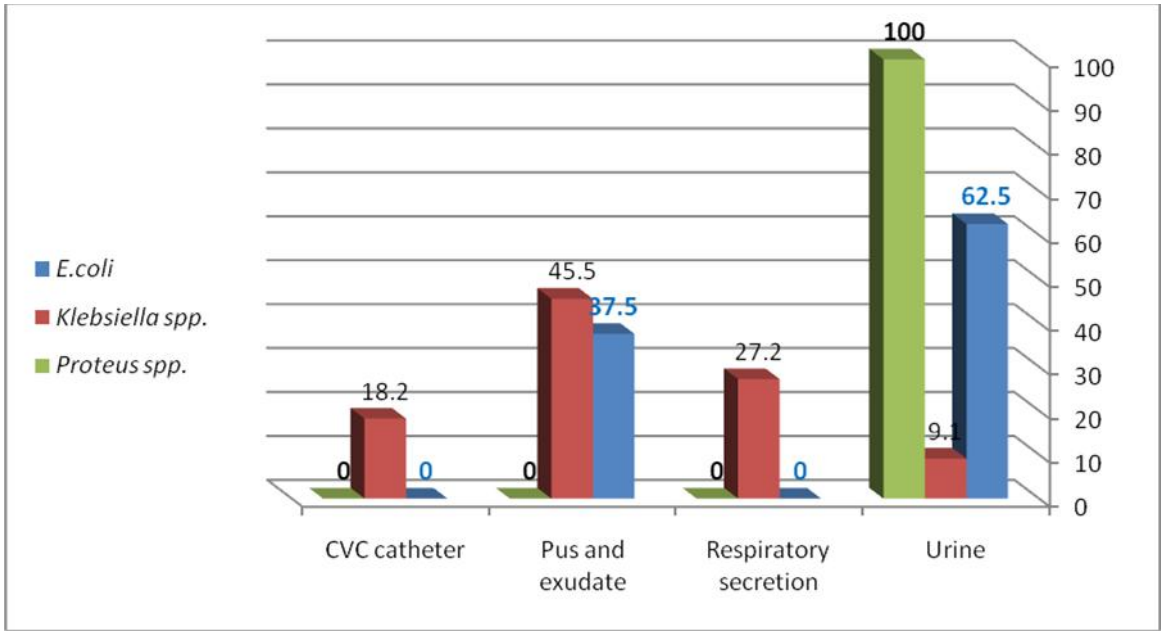
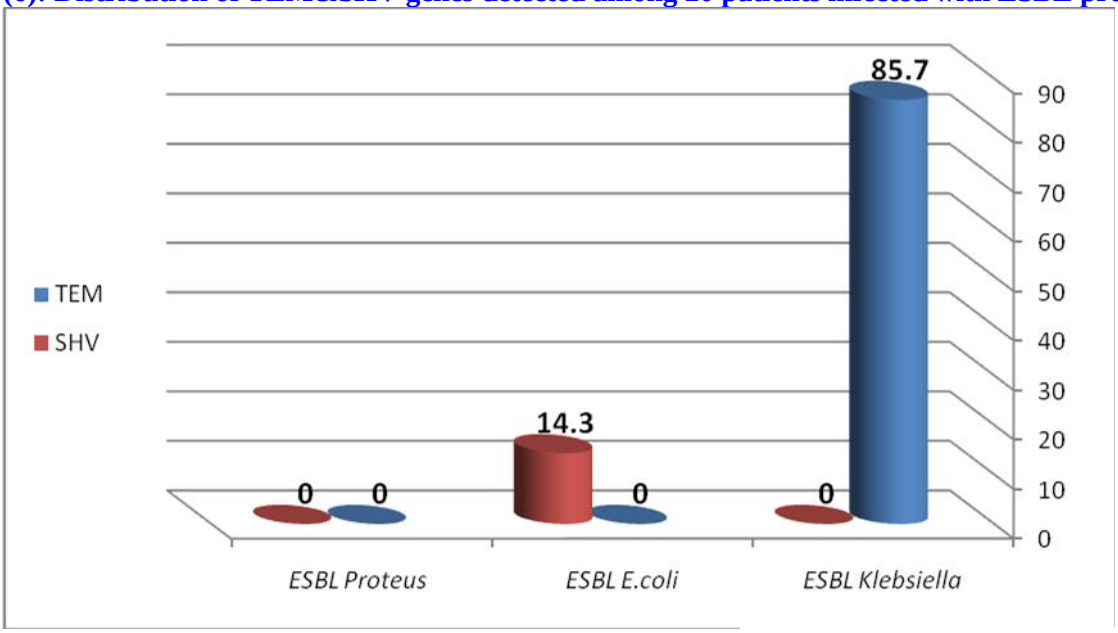


Figure (6): Distribution of TEM&SHV genes detected among 20 patients infected with ESBL producer.



Discussion

Antimicrobial resistance is a major global problem in both developing and developed countries. The emergence of multi drug resistant (MDR) bacteria may be the greatest concern for HAI's in ICUs, not only due to increased morbidity and mortality, but also due to increased treatment costs as a result of frequent empirical failure and lengthy hospital stay. Indeed, more than 70% of critically ill patients will be given an antimicrobial drug during their ICU stay. (Alanis, 2005; Vincent et al., 2009) ICU is deemed the epicenter of resistance development; it has even been described as a factory for creating, disseminating, and amplifying antimicrobial resistance. (Alanis, 2005; Petrosillo et al., 2010)

In the present study, 250 samples were examined. Of these samples 188(58.1%) yielded single isolates and 94 (29.01%) revealed mixed isolates. Goel et al, in India, demonstrated that the majority of isolates from lower respiratory tract of ventilated patients were single ones 127/161(78.8%). (Goel et al., 2009) In addition, the results of the present work revealed a statistical significant difference (p=0.006) between the frequencies of single and mixed isolates as regards Gram negative, Gram positive and fungal isolates (77.1% and 66.9%),(22.0% and20.2%),and (11.0% and 2.6%),respectively. This was in accordance with other studies. (Husičková et al., 2013; Vandijck et al., 2008; Wasnik, 2013)

It was prominent in the current study that the majority of isolates were Gram negative bacteria 236/324 (72.8%).In King Fahad National Guard Hospital ICU, Al Johani et al (2010), reported nearly similar findings to that revealed in this study (66.6%), (Al Johani et al., 2010) while relatively lower rates were demonstrated by Zahid et al., (2009) in Pakistan, and Lee et al., (2009) in India representing 57.6% and 56.2%, respectively. (Zahid et al., 2009; Lee et al., 2009)

On the other hand, higher percentages were reported by Goel et al., in 2009 (95.6%) and khan, in 2012 (85.0%) in Saudi Arabia. A variety of factors may account for the variation between one study and another, including demographic and clinical characteristics, differences in methods of sampling, misdiagnosis, and the use of antimicrobial therapy. (Goel et al., 2009; Khan, 2012)

K. pneumoniae is the most important and most common infectious pathogen in hospitals environment and is mainly responsible for pneumonia, UTI, neonatal septicemia and wound infections among children. (Ndugulile et al., 2005) This coincides with the present results, where *K. pneumoniae* was the most frequent represented Gram negative organism 26.2%, followed by *E.coli* (23.1%), *P.aeruginosa* (13.8%) and *Acinetobacter baumanii* (7.4%).

Khan, in a study to evaluate the microbiological spectrum and susceptibility pattern of pathogens in ICU, demonstrated that *Acinetobacter baumanii*, *Klebsiella*, and *P.aeruginosa* were the most common isolates among Gram negative organisms (24.0%, 22.0%, 20.0%), respectively. (Khan, 2012) The author of the latter study attributed his finding to the fact that most isolates were recovered from the respiratory samples.

As regards Gram positive isolates in the present study, CoN*S* encountered the highest percentage (14.2%), followed by *S.aureus* (5.6%), while MRSA was found only in 0.9 % of these isolates. This agrees with Khan's findings who demonstrated that CoNS and *S. aureus* were the two leading Gram positive isolates (8.5%, 12.4%), respectively (Khan, 2012) Similar results were obtained by Ndugulile et al. and Zahid et al. The vast variations in the frequency of isolation of different pathogens between hospitals is most probably due to the variation in patient populations, the applied antibiotic regimen, departments in each hospital, and subsequently the type of specimens sent to laboratories. (Ndugulile et al., 2005; Zahid et al., 2009)

The high prevalence of CoNS isolates is alarming that special attention should be given to controlling the dissemination of these opportunistic bacteria in ICU patients. Appropriate antibiotic therapy and control measures could be adopted to prevent cross contamination of multidrug-resistant CoNS bacteria from previous ICU patients to new patients and hospital staff. (Ejaz et al., 2013)

Due to their immunocompromised status, patients in the ICU are at risk of invasive candidiasis. (Brusselaers et al., 2011; Miceli et al., 2011) The problem of MDR in candidiasis merely results from a shift in etiology from mainly *C. albicans* to non-*albicans* spp., Leroy et al. (Leroy et al., 2009) found that almost half of the invasive candida infections in the ICU (N = 300) were due to non-albicans species and reduced susceptibility to fluconazole was observed in 17% of all *Candida* isolates. However, all the 20 candida spp. recovered in the current study, were *C. albicans*.

UTIs are the most frequent infections worldwide among hospitalized patients, and Enterobacteriaceae (mainly *E.coli*) are generally the causal agents. In the current study, the main pathogens involved in UTI were *E. coli* (56.8%) followed by *K. pneumoniae* (18.7%) and *P.aeruginosa* (11.3%). Similar rank order for pathogens causing UTI was found in a study conducted in Makah, in Saudi Arabia: 56.8% for *E. coli*, 18.6% for *Klebsiella* and 16.1% for *P. aeruginosa*. (Leroy et al., 2009 ; Asghar et al., 2009)

Many researchers had declared that *E.coli* was the most commonly encountered isolate from urine samples.(Meric et al., 2005; Japoni et al., 2009; Khalili et al., 2012) It is to be expected that *E. coli* is the common colonizing or infecting agent of the UT. This nearly agrees with Batchoun et al., who reported that *E.coli* was the most common isolated organism from urine samples, representing (41.4%) of the total isolates from three teaching hospitals in Northern Jordan, followed by *K. pneumoniae* which is considered the second isolated pathogen where it constituted 25.0%. On the other hand, *P.aeruginosa* was the most common organism isolated from swabs from various sources (18.8 %). (Batchoun et al., 2009)

In a general hospital in San Fernando, Orrett reported that the predominant isolates from culturing urine samples were *P.aeuroginosa* and *Klebsiella*. In addition he reported that regarding sputum samples; *P.aeruginosa* and *K. pneumoniae* were the most common isolates. This coincides with the findings of the present study where the most common isolated Gram negative bacteria in sputum samples were *Klebsiella* and *P.aeruginosa* (40.7%, and 32.5%, respectively). (Orrett, 2004)

Antibiotics are the most frequently prescribed drugs among hospitalized patients especially in ICU and surgical departments. Several studies have reported concern about the continuous indiscriminate and excessive use of antimicrobial agents that promote the emergence of antibiotic-resistant organisms. Monitoring of antimicrobial use and knowledge of prescription habits are some of the strategies recommended to contain resistance to antimicrobials in hospitalized patients. (Behzadia et al., 2010; Brito et al., 2006; Badar et al., 2012)

In the current study, the most commonly used antibiotics belonging to penicillins, cephlosporins, fluoro-quinolones, aminoglycosides, quinolones, glycopeptides, carbapenems and lincosamides were tested against the bacterial isolates to know the current status of the resistance pattern. The results of antimicrobial susceptibility revealed that *E.coli* showed a high sensitivity to carbapenems and amikin (100.0%) each and moderate sensitivity to cephalosporins and β-lactam/ β- lactamase inhibitor combinations ranging from 80.0% to 89.3%. High sensitivity to amikin and carbapenems was noticed in *K. pneumoniae* (100, 0%, 94.1%), respectively, while low sensitivity was observed to ampicillin-sulbactam 54.1%. Regarding cephalosporins, high sensitivity was for cefoperazone and cefipime (92.9%, 90.5%), respectively. Moreover, low sensitivity was for quinolones in *P.aeruginosa* with maximum percentage 77.6%, while high sensitivity was noticed in carbapenems 86.6%. Sensitivity to each of cefipime and cefazolin was found in 84.4 % of *P.aeruginosa* isolates in this study.

As for *Acinetobacter baumanii*, all the 24 isolates showed high sensitivity to amikin (100.0%), and 83.3% were sensitive to each of gentamicin, imipenem and meropenem, while cephalosporins group showed low effect on *Acinetobacter baumanii*, sensitivity ranged from 38.0% to 50.0 %.

Acinetobacter is an increasingly infectious threat, especially for patients receiving broad spectrum antimicrobial therapy and requiring life support. (Goel et al., 2009) A Spanish study has shown that *Acinetobacter* isolates, usually acquired in the ICU, are MDR and may cause severe infections associated with a high mortality rate. It is an important source of nosocomial septicemia, pneumonia, and UTIs. (Khan, 2012). Reports of MDR isolates have increased during the last decade, probably as a result of the extensive use of broad-spectrum antibiotics. (Warren et al., 2005; Horan and Gaynes, 2004) In many cases, these MDR isolates are resistant to expanded-spectrum cephalosporins and carbapenems.(Horan and Gaynes, 2004; Cisneros et al.,2002)

The prevalence of ESBL producing organisms varies from one country to another and from institution to institution with low rates of 3-8% reported in Sweden, Japan and Singapore compared to much higher prevalence rates documented in studies from Portugal (34%), Italy (37%), New York (44%), Latin American countries (30-60%) and Turkey (58%). (El-Khizzi and Bakheshwain, 2006) Within the Arabian Gulf region, high ESBL prevalence of 31.7% in Kuwait and 41% in the United Arab Emirates has been reported among inpatients. For Saudi Arabia, reported ESBL rates varied from 8.5-38.5% . (El-Khizzi and Bakheshwain, 2006; Khanfar et al., 2009; Kader and Angamuthu, 2005; Kader and Kumar, 2004; Panhotra et al., 2004) Thus in comparison to these data, the finding of 8.5% ESBL producers in the current study is on the lower end of the spectrum. This finding is also similar to data reported from surveys in some countries in Europe and Asia. (Perez et al., 2012; Daza et al., 2001)

According to Riaz et al., findings, high percentage of ESBL producing bacteria was among males (64%) compared to females who represented 36%. (Riaz et al., 2012) Regarding this study it was on the same line with the present study that the percentage of ESBL producers among males was higher than ESBL producers among female patients especially in age group 60 years and more.

Regarding co-morbidity risk factors, in the current study, DM and pneumonia were the most frequent ones (25.0% each), followed by malignancies and renal impairment (20.8% and 15.0%, respectively). These results consisted with the findings of Rubio-Perez et al., who concluded that DM was the most frequent co-morbidity, present in 33.0 % of their hospitalized patients in ICU. They attributed their findings to the altered metabolism and associated immune deficiency that may have led to the higher risk of infection among diabetic patients, particularly those related to wound, catheter and bacteremia. (Rubio-Perez et al., 2012)

Patients in critical care units are likely to have higher use of invasive devices such as urinary and vascular catheters. (Tumbarello et al., 2011) It was noticed from this study, that the highest percentage of ESBL producing bacteria was among patients who had indwelling urinary catheters (20.0%), followed by patients with CVC (15.0%). Khanfar et al. found that indwelling urinary catheter was the major source of ESBL isolates (52.2%).

The present study illustrated the distribution of TEM and SHV genes among Enterobacteriaceae, where TEM was the most commonly detected gene in *K. pneumoniae* (85.7%), while only SHV was found in *E.coli* (14.3%). No genes were detected in *Proteus* spp. Feizabadi et al., reported that the prevalence of genes encoding ESBLs were common in *Klebsiella* spp, TEM (54.0%) and SHV (67.4%). (Feizabadi et al., 2010) Jain and Mondal, reported that 75.0% of *Klebsiella* spp. revealed bla $_{TEM}$ gene, while bla $_{SHV}$ gene was found in 46.8%, while 26.5 % had both bla $_{TEM}$ and bla $_{SHV}$ genes (Jain and Mondal , 2007)Ahmed et al., reported that PCR for TEM and SHV revealed that both genes were common in *Klebsiella* spp. *(*58.0% and 63.1%, respectively). (Ahmed et al., 2013)

Bali et al., found that TEM type ESBLs genes were the most common genes detected in *Klebsiella* (73.33%), followed by *E. coli* (72.72%) .On the other hand, they found that SHV type ESBL was frequently found in *Klebsiella* spp (53.3%). (Bali et al., 2010)

In the present study, the rate of detection of TEM type ESBLs genes in *Klebsiella* was more than that reported in Tasli and Bahar study (83.3% and 84.1%, respectively). (Tasli and Bahar 2005) However a nearly similar percentage was reported by Al-Agamy et al. (84.1%).(Al-Agamy et al., 2009) This high percentage may reflect aggressive behavior of these strains.

The results of this study showed that the prevalence rate of ESBLs, β-lactamase genes and resistance to multiple antibiotics were noticeable among Enterobacteriaceae

isolates, especially *E. coli* and *K. pneumoniae*. Physicians should pay attention to the fact that using of many ineffective antibiotics and possibility of ESBL genes spreading between different species of Enterobacteriaceae will help in the dissemination of ESBL-producing isolates. (McDonnell, 2008; Marcel et al., 2008)

Determination of TEM and SHV in ESBL producing bacteria may give useful data about their epidemiology and risk factors associated with these infections. Therefore, ESBL producing organisms should be promptly identified for appropriate antibiotic prescription and proper implementation of infection control measures. (Javadian et al., 2014)

Conclusions

From the results of this study it could be concluded that:

1. The majority of isolated pathogens were single.
2. Gram negative bacilli were the most frequently isolated pathogens, with the highest percentage for *Klebsiella* spp.
3. *E.coli* was the most frequently encountered isolate in urine and pus and exudate samples.
4. *Klebsiella* spp. was the most prevalent ESBL producer.
5. 5.Carbapenems group showed high sensitivity among Gram negative bacteria, while glycopeptides had strong effect on *S.aureus* and MRSA.
6. Advanced age (60 years and more) and male gender are accepted risk factors for infection by ESBL producers.
7. DM and pneumonia were the most frequent co-morbidity risk factors among ESBL producer patients.
8. TEM was the most commonly detected gene in *Klebsiella pneumonia*e.

Recommendations

1. The recognition of the risk factors for infection by ESBLs could aid in the identification of patients at high risk of harboring ESBL producing pathogens, thus enabling administration of more efficient empiric antibiotic treatment.
2. Due to the increasing antimicrobial resistance rate in hospitals, antimicrobial susceptibility testing should be routinely employed to ensure appropriate antibiotic prescription, in an attempt to decrease antimicrobial resistance among critically ill patients.
3. Laboratory methods for detection of ESBL producing pathogens should be done routinely for early diagnosis of these organisms especially among critically ill patients.

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