Prevalence and Characterization of Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae in Brack-Alshati, Fezzan, Libya

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Abstract

Background: Extended spectrum β-lactamase ESBL-producing Enterobacteriaceae are increasing worldwide. However, there is sparse data on their prevalence and nature in Libya. A prospective study was performed to:

a. Assess the prevalence of ESBL-producing Enterobacteriaceae among gastrointestinal and urinary tract infection patients and healthy individuals, and
b. Determine the antibiotics sensitivity profiles and β-lactamase production of the isolates.

Methods: 307 individuals were voluntarily enrolled in this study; 201 patients and 106 healthy individuals. 106 stool samples were collected from the healthy group and 80 stool and 121 urine samples were from patients. Enterobacteriaceae isolates were tested for ESBL production by the double disk synergy test, and antibiotic susceptibility testing was performed using the disk diffusion method.

Results: Of the total of 333 bacterial isolates, 208 were from patients and 125 from the healthy group. Patients yielded 149 E. coli (71.6%), 35 Klebsiella pneumoniae (16.8%), 6 Klebsiella oxytoca (2.9%), 6 Proteus mirabilis (2.9%), 5 Enterobacter cloacae (2.4%), 4 Citrobacter freundii (1.9%), 2 Enterobacter agglomerans (0.96%) and 1 Salmonella typhi (0.48%). On the other hand, 91 E. coli (72.8%), 24 K. pneumoniae (19.2%), 5 Ent. cloacae (4%) and C. freundii (4%) isolates were from the healthy group. In total, only 15 isolates (4.5%) produced ESBL of which, 12 (5.7%) were from patients and 3 (2.4%) from the healthy group. 8 out of 149 (5.36%) E. coli, and 4 out of 35 (11.4%) K. pneumoniae produced ESBLs from patients’ isolates compared to a single E. coli (1.09%) and 2 K. pneumoniae (8.3%) from the healthy group isolates.

Conclusions: The prevalence of ESBL-producing Enterobacteriaceae strains was 4.5% among isolates from healthy and patients stool and urine samples in the targeted area in Libya. ESBL strains were more prevalent in patients’ samples (5.7%) than healthy controls (2.4%). Their antibiotic sensitivity profile showed more resistance in patients’ isolates than the healthy volunteers.

Keywords: Extended β-lactamase-producing; Enterobacteriaceae; E. coli; K. Pneumoniae; Antibiotic resistance; Libya

Abbreviations: AML: Ampicillin; AMP: Amoxicillin; TIC: Ticarcillin; KF: Cephalothin; FOX: Cefoxitin; CFP: Cefoperazone; CTX: Cefotaxime; CAZ: Ceftazidime; CRO: Ceftriaxone; IPM: Imipenem; ATM: Aztreonam; TZP: Piperacillin/Tazobactam; AMC: Amoxicillin/Clavulanic acid (augmentin); CIP: Ciprofloxacin; NA: Nalidixic acid; F30: Nitrofurantion; C30: Chloramphenicol; TE: Tetracycline; SXT: Trimethoprim/Sulfamethoxazole; CN: Gentamicin

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Introduction

The increase in antimicrobial resistance of pathogenic bacteria is a major problem worldwide. Extended spectrum β-lactamases (ESBLs) are plasmid-encoded enzymes that are produced most often by *Klebsiella* and *E. coli* species. ESBLs are the most important factor contributing to Gram negative bacilli resistance to broad-spectrum β-lactam antibiotics such as cefotaxime, ceftriaxone, ceftazidime, aztreonam and cefpodoxime [1]. These β-lactamases are easily transferable among bacterial species. They are mostly of the KPC, VIM, IMP, NDM, and OXA-48 types. Their current extensive spread worldwide in Enterobacteriaceae is of great importance and concern. Infections caused by these bacteria have limited treatment options and have been associated with high mortality rates. The prevalence of carbapenemases in the Mediterranean region is particularly high constituting one of the most important reservoirs [2]. The first ESBL was reported in *Klebsiella pneumoniae* in 1983 [3]. The majority of ESBLs isolated from clinical samples have been SHV or TEM types of TEM-1, and 2, and SHV-1 [4]. More than 40 of these extended spectrum enzymes (named TEM-1, -2, -3 and SHV-1, -2, etc) have been discovered since [4]. These ESBLs have enhanced stability in the presence of β-lactam antibiotics and are positively selected through point mutations in the TEM and SHV β-lactamase genes [5,6]. In recent years, β-lactamases that hydrolyze carbapenems have also been discovered, such as; the *Klebsiella pneumoniae* carbapenemase (KPC) and metallo-β-lactamases (MBLs), such as the New Delhi metallo-β-lactamase (NDM) [7,8].

ESBL-producing Enterobacteriaceae have been responsible for numerous outbreaks of infections throughout the world and pose challenging infection control measures [9]. Urinary tract and blood stream infections are some examples. Intravascular and urinary catheters, emergency intraabdominal surgery, gastrostomy or jejunostomy tube, gastrointestinal colonization, length of hospital or intensive care unit stay, prior antibiotics, prior nursing home stay, severity of illness, and ventilator assistance are some risk factors for acquiring an infection with ESBL producing strains [10,11]. The frequency of ESBL-producing bacteria differs significantly by population geographic location [12-15].

Although the study of ESBL-producing Enterobacteriaceae in Libya was previously attempted [16,17], however, none has attempted to investigated samples from rural areas. Therefore, the goal of this study was to investigate the prevalence of ESBL-producing Enterobacteriaceae among gastrointestinal and urinary tract infection patients and healthy individuals in the southern Brack-Alshati area, Fezzan, Libya. Their antibiotic sensitivity profile to a panel of antibiotics employing double disk synergy and disk diffusion test, and β-lactamase production along with other isolates was determined.

Materials and Methods

This study voluntarily enrolled 307 individuals comprising 201 patients-aging 7 months to 95 years attending Brack General Hospital and other private clinics in this area and 106 healthy controls aging 19 to 36 years. The samples were collected in the period from October 2010 to April 2011. The male to female ratio was 1 : 2 among patients and controls. Healthy controls provided 106 stool samples and 80 stools and 121 urine samples were collected from the patients. An informed consent was obtained from all participants or their custodians prior the participant enrollment and the study was approved by our local committee for Medical and Research Ethics.

All samples were inoculated onto MacConkey agar plates (Mast Diagnostic, Mast Group LTD, Merseyside, UK). Additionally, the stool samples were inoculated onto Xylose Lysine Deoxycholate (XLD; Oxoid Limited, Hants, England). All plates were incubated at 37°C for 24 hours in air. The suspected colonies were streaked onto fresh MacConkey agar plates for purity and later identified using standard microbiological methods- Gram staining, oxidase test, IMVIC and carbohydrate fermentation tests. Isolates identified as Enterobacteriaceae were finally preserved on nutrient agar slants in individual screw capped bottles at 4°C.

The antibiotic susceptibility testing was performed using the disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI) guidelines [18]. *E. coli* ATCC 25922 was used as a control. Commercially available antibiotic discs (Oxoid Limited, Basingstoke, Hampshire, England) were used for antibiotics susceptibility testing including that included Ampicillin (AML, 10 µg), Amoxicillin (AMP, 10 µg), Ticarcillin (TIC, 75 µg), Amoxicillin/Clavulanic acid (Augmentin) (AMC, 20/10 µg), Piperacillin/Tazobactam

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(TZP, 100/10 µg), Cephalothin (KF, 30 µg), Cefoperazone (CFP, 5 µg), Cefoxitin (FOX, 30 µg), Cefotaxime (CTX, 30 µg), Ceftriaxone (CRO, 30 µg), Ceftazidime (CAZ, 30 µg), Imipenem (IPM, 30 µg), Aztreonam (ATM, 30 µg), Gentamicin (CN, 10 µg), Tetracycline (TE, 30 µg), Ciprofloxacin (CIP, 5 µg), Nalidixic acid (NA, 30 µg), Chloramphenicol (C, 30 µg), Nitrofurantoin (F, 300 µg), and Trimethoprim/Sulfamethoxazole (SXT, 1.25/23.75 µg).

The one-minute β-lactamase test was performed as described [19] to test for β-lactamase production by the bacterial isolates using the double disk synergy technique modified by Lautenbach, et al. [13]. Briefly, a single colony from an overnight blood agar culture was suspended in sterile 0.9% normal saline solution in a sterile tube. The turbidity of the test bacteria was adjusted by dilution to obtain 0.5 McFarland standards. A cotton swab was soaked in the suspension tube and the test bacteria were spread on Mueller-Hinton agar plates (Oxoid Limited, Hants, England). The disks containing the standard ceftazidime, ceftriaxone, cefotaxime, and aztreonam at 30 µg were placed 25 mm apart (center to center) from amoxicillin/clavulanic acid 20/10 µg. After an incubation period of 18 hours at 35°C, the zone of inhibition between Ceftazidime, Ceftriaxone, cefotaxime or aztreonam and amoxicillin/clavulanic acid indicated the presence of an ESBL-producing isolate. E. coli ATCC 25922 was used as negative control.

Results

A total of 333 bacterial isolates were isolated from patients and healthy individuals. Of these, 208 strains were isolated from patients and 125 were isolated from the healthy volunteers. Out of the 208 bacterial strains isolated from patients, 149 were Escherichia coli (71.6%), 35 were Klebsiella pneumoniae (16.8%), 6 were Klebsiella oxytoca (2.9%), 6 were Proteus mirabilis (2.9%), 5 were Enterobacter cloacae (2.4%), 4 were Citrobacter freundii (1.9%), 2 were Enterobacter agglomerans (0.96%) and 1 was Salmonella typhi (0.48%). On the other hand, of the total 125 isolates from the healthy volunteers, 91 were E. coli (72.8%); 24 were K. pneumoniae (19.2%); 5 were Ent. cloacae (4%) and 5 were C. freundii (4%).

<table>
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<th>Patients</th>
<th></th>
<th>Healthy Controls</th>
<th></th>
<th>Total</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Isolates</td>
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<td>Isolates</td>
<td>ESBL (%)</td>
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<td>ESBL (%)</td>
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<td>2 (8.3)</td>
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<tr>
<td>Ent. agglomerans</td>
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<tr>
<td>Total</td>
<td>208</td>
<td>12 (5.8)</td>
<td>125</td>
<td>3 (2.4)</td>
<td>333</td>
<td>15 (4.5)</td>
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</tbody>
</table>

Table 1: Distribution of extended-spectrum β-lactamase producing and non-producing Enterobacteriaceae isolated from patients fecal and urine samples and fecal healthy control samples collected from Brack-Alshati, Fezzan, Libya.

Out of the total 333 Enterobacteriaceae isolates from both patients and healthy controls, only 15 (4.5%) isolates were positive for ESBL as tested by the double disc synergy test. Among those 15 isolates, 12 (3.6%) isolates were from patients and 3 (0.9%) isolates were from healthy volunteers. In patients, 8 (5.36%) out of 149 E. coli strains, and 4 (11.4%) out of 35 K. pneumoniae strains were ESBL-producers. However, only a single (1.09%) E. coli strain out of 91 and 2 (8.33%) K. pneumoniae strains out of 24 isolated from healthy group produced ESBL. Of the ESBL-producing strains, 11 of patients’ 12 isolates were from urine samples. Almost all the ESBL-producing isolates were consistently more resistant to a wide variety of antimicrobial agents than the non-ESBL-producers. The most

distinguishing feature of ESBL-producing *E. coli* and *K. pneumoniae* was their higher level of resistance to ampicillin, amoxicillin, ticarcillin, cephalothin compared to the non-ESBL-producing isolates. ESBL-producing *E. coli* isolates were also resistant to third generation cephalosporins such as ceftazidime, ceftriaxone, and cefotaxime with a range of 11-33%, while non-ESBLs *E. coli* isolates were sensitive to these drugs.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>K. pneumoniae</em></th>
<th>% Resistance</th>
<th><em>E. coli</em></th>
<th>% Resistance</th>
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<td>Non-ESBL</td>
<td>ESBL</td>
<td>Non-ESBL</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>85</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td>Amoxicillin</td>
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<td>85</td>
<td>100</td>
<td>45</td>
</tr>
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<td>Ticarcillin</td>
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<td>85</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>83</td>
<td>15</td>
<td>100</td>
<td>42</td>
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<tr>
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<td>15</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>Cefoperazone</td>
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<td>22</td>
<td>3</td>
</tr>
<tr>
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<td>0</td>
<td>33</td>
<td>0</td>
</tr>
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<td>0</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Ceftriazone</td>
<td>50</td>
<td>0</td>
<td>33</td>
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<tr>
<td>Piperacillin/Tazobactam</td>
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<td>22</td>
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<td>Amoxicillin/clavulanic acid</td>
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<td>Ciprofloxacin</td>
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<td>4</td>
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<tr>
<td>Nalidixic acid</td>
<td>17</td>
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<td>Nitrofurantion</td>
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<td>3</td>
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<tr>
<td>Choramphenicol</td>
<td>50</td>
<td>11</td>
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<td>Tetracycline</td>
<td>50</td>
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<td>42</td>
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<td>Trimethoprim/Sulphamethaxzole</td>
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<tr>
<td>Gentamicin</td>
<td>33</td>
<td>0</td>
<td>55</td>
<td>3</td>
</tr>
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</table>

**Table 2**: Comparison of resistance patterns of extended-spectrum β-lactamase-producing and non-producing *K. pneumoniae* and *E. coli* isolated from patients fecal and urine samples and fecal healthy control samples collected from Brack-Alshati, Fezzan, Libya to antibiotics.

The 15 ESBL-producing isolates showed variable resistance patterns. The resistance to cefotaxime, ceftriaxone, ceftazidime, and aztreonam were 40%, 40%, 26.7% and 6.7%, respectively. The correlation between the zone of inhibition recorded for the five antibiotics (ceftazidime, cefotaxime, ceftriaxone, aztreonam and augmentin) tested with the disk diffusion test and the zone of inhibition of the same antibiotics used above with the double disk synergy test (DDS, 25 mm) have revealed a positive correlation for both *E. coli* (r=95.2%, p=0.001) and *K. pneumoniae* (r=88.3%, p=0.001) as shown in Figure 1, suggesting equal validity for the two methods.

The results of β-lactamase testing revealed that 119 of the *E. coli* isolates (80%), 52 of *K. pneumonia* (88%), 9 of *Ent. cloacae* (90%), 5 of *K. oxytoca* (83.3%), 3 of *C. freundii* (33.3%), and 1 of *Ent. agglomerans* (50%) did not produced β-lactamase. Among *E. coli* isolates, 9 strains (3.8%) were positive for ESBL production. Similarly, among the *K. pneumoniae* isolates, six strains (10.2%) were positive for both.

*K. pneumoniae* isolated from patients showed higher levels of resistance against almost all the antimicrobials tested compared to *K. pneumoniae* isolates from the healthy volunteers.

**Figure 1:** Correlation of the result of the two methods: Double Disk Synergy (DDS) and Disk Diffusion Test (DDT) for detection of β-lactamase production in *K. pneumoniae* (Panel A) and *E. coli* (Panel B) isolated from patients fecal and urine samples and fecal healthy control samples collected from Brack-Alshati, Fezzan, Libya (p = 0.00).

All 39 (100%) *K. pneumoniae* isolates from patients were resistant to ampicillin, amoxicillin and ticarcillin. In contrast, only 67% of *K. pneumoniae* strains isolated from the healthy group were resistant to the same antibiotics (p < 0.05). A similar pattern was seen among *E. coli* isolates from patients that showed high level of resistance to the majority of the antimicrobials compared to *E. coli* isolates from the healthy group.

**Figure 2:** Comparison of antibiotics resistance of *K. pneumoniae* (panel A) and *E. coli* (panel B) isolated from patients fecal and urine samples (light shaded bars) and fecal healthy control samples (dark shaded bars) collected from Brack-Alshati, Fezzan, Libya.

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For instance, a range of 48-60% of isolates were resistant to the commonly used drugs including ampicillin, amoxicillin, ticarcillin, cephalothin and tetracycline compared with resistance rates for the healthy group (p < 0.05). Other antibiotics including cefoxitin, ceftriaxone, augmentin, nalidixic acid and trimethoprim/sulphamethaxzole showed variable antimicrobial efficacy against isolates from both patients and healthy group with no statistical significance difference for each of E. coli and K. pneumoniae.

Discussion

The prevalence of Enterobacteriaceae strains producing ESBLs varies worldwide. The highest rates among K. pneumoniae isolates from clinical specimens were reported from Latin America (44%), Asia/Pacific Rim (22.4%), Europe (13.3%), and North America (7.5%). A similar trend was shown for E. coli in terms of specific geographical variation but at lower prevalence rates of 13.5%; 12%; 7.6% and 2.2%, respectively [12]. In our study, a much lower rate of 4.5% of all isolates-from both patients and healthy volunteers were ESBL producers. Such rate was higher considering patients’ samples only (5.8% ESBL-producers). This is comparable to 6.3% reported by Bonfiglio in Italy [20]. A higher percentage of 13.4% among E. coli isolates from diarrhea stool samples of Northern Libyan children has also been recorded; while nine E. coli isolates (6.7%) demonstrated AmpC β-lactamases [17]. However, this prevalence is lower than those reported in other countries. For instance, it was reported that the prevalence was 55% in Jordan [21], 42% in Israel [22], 36% in Saudi Arabia [23], 38.5% in Egypt and 27.4% in Greece [24]. Countries of the Gulf Cooperation Council share a high prevalence of ESBL-and carbapenemase-producing Gram-negative bacilli, most of which are associated with nosocomial infections [25]. Infections with bacteria producing extended-spectrum beta-lactamases (ESBLs) are increasing across Africa too. ESBL-producing Enterobacteriaceae are significant causes of infections and antibiotic resistance at Korle-Bu Teaching Hospital in Accra, Ghana with ESBL expression rate of 49.3% in clinical samples [26].

Nonetheless, lower prevalence rates were also reported from other countries such as Denmark with less than 1% prevalence in the Copenhagen area [27]. Variability in the reported ESBL production prevalence could be attributed to a number of factors. For most of these types of studies, bacteria were isolated from ICUs patients and other hospitalized patients. Furthermore, studies looking for ESBL prevalence are usually performed on bacterial isolates from nosocomial infections. This in return may favour the finding of Enterobacteriaceae strains that produce ESBL and hence inflating the percentage of prevalence of ESBL-producing bacteria [12,28-30]. Antibiotic policies and protocols implemented at different geographical regions may also contribute to these differences in prevalence [6,9, 31,32]. A Saudi study reported 12.7% rate of ESBL expression among isolates from fecal specimens of healthy individuals and community outpatients. Of these, 87 (95.6%) were E. coli and 4 (4.4%) Klebsiella pneumoniae [30]. The community could be a reservoir of these ESBL-producing bacteria because rate of fecal carriage of ESBL-producers was very comparable among healthy persons (12.3%) and community outpatients (13.7%). The extensive usage of antibiotics, particularly non-prescription empirical usage, may correlate with greater prevalence due to positive selection. Moreover, environmental and genetic background susceptibility factors could have a significant impact on prevailing microbial ecology [33].

The prevalence of ESBL was greater among K. pneumoniae strains compared to E. coli in the present investigation. This is in agreement with previous studies [20-22,34-36]. Out of 125 isolates from the healthy volunteers, ESBL production was found only in 3 (2.4%) strains. Although a very low prevalence, healthy carriers of ESBL-producing bacteria may act as a reservoir for the spread of multi-drug resistant bacteria in the community. In general, strains of E. coli and K. pneumoniae that produced ESBLs were more resistant to the β-lactam and non-β-lactam antibiotics tested than non-ESBL expressing strains. Only imipenem was effective against both groups. This was in agreement with a number of other reported studies [24,37,38]. The susceptibility pattern for piperacillin/tazobactam against ESBL-producing bacteria has been extremely variable. In the present investigation, the sensitivity rates of both ESBL-and non-ESBL-producing strains of K. pneumoniae ranged from 94-100%. This is in agreement with the findings at Asia-Pacific region and South Africa medical centers, and other studies [3,39-41]. However, ESBL-producing E. coli strains have shown a greater resistance to

piperacillin/tazobactam (22%) than non-ESBL-producing E. coli strains (0%). In a Saudi study, prevalence of ESBL was 25.6% among isolates of K. pneumoniae and all ESBL-positive isolates were sensitive to imipenem and tigecycline. However, the resistance rate to gentamicin, amikacin, and ciprofloxacin was 87.3%, 10%, and 9.1%, respectively [42]. A hospital-based isolation would show higher prevalence of ESBL-production that was the case for a study conducted at Riyadh, Saudi Arabia showing sticking high rate of ESBL-producing K. pneumoniae isolates (55%) [43]. However, in the same city 20.4% of E. coli isolates were ESBL-producers [44]. While an Iranian clinical isolates of E. coli showed 33.3% were ESBL-producers [45]. In another clinical Enterobacteriaceae study in the Netherlands ESBL gene expression was evident in 81% of isolates [46].

Although, it has been reported that gentamicin can be used against ESBL-producing bacteria [41], we have found that 55% of ESBL-producing E. coli were resistant to gentamicin, compared with 3% of the non-ESBL-producing E. coli. Similarly, 33% of ESBL-producing K. pneumoniae were resistant to gentamicin, whereas, none of the non-ESBLs-producing isolates were resistant to the same drug. These findings are in agreement with other data reported from UK [47]. Nonetheless, another report [40], suggested that ESBL-producing strains may not necessarily be more susceptible to gentamicin. A Ghanaian study showed marked increase in minimum inhibitory concentrations of ESBL-producing Enterobacteriaceae isolated from clinical samples compared with other strains. The study found that 17% of ESBL-producers were resistant to two or more antibiotics (aminoglycosides, fluoroquinolones, sulfonamide, and carbapenems), whereas, only 3.2% of non-ESBL-producers were multidrug resistant [26].

It is a well known fact that using the disk diffusion test as a single method for detection of ESBL expression is not adequate. Other investigators have reported similar observations [20,21,48-50]. ESBL-producers are able to hydrolyze extended-spectrum penicillins, cephalosporins and aztreonam. The minimal inhibitory concentration (MICs) of these antimicrobial agents may be within the sensitivity range. For this reason, CLSI guidelines recommends that ESBL-producers be reported as resistant to all penicillins and cephalosporins and aztreonam, even when they show sensitivity to these agents by such conventional tests [18]. A positive significant correlation between the two antimicrobial sensitivity methods was evident for E. coli and K. pneumoniae. Therefore, we and other investigators recommend the use of the double disk synergy in conjunction with the disk diffusion test for the detection of ESBL-producing bacteria in the laboratory setting [51].

According to the present results, strains isolated from patients were more resistant to all antibiotics tested in comparison to those isolated from healthy individuals. A similar observation was noted by Kunin and Liu who recorded high frequency of antibiotics use and resistance among patients presenting at emergency wards, clinics and the community in Taiwan [52], and in patients from the Gulf area [53]. However, there were few exceptions where K. pneumoniae isolates were more resistant to cefoxitin and nitrofurantoin. C. freundii was more resistant to augmentin, while Ent. cloacae isolates were more resistant to ampicillin, amoxicillin, cefoxitin, cephalothin and nitrofurantoin in the healthy group of individuals in the present study . The differences in the overall antibiotic resistance of E. coli and K. pneumoniae in patients and the healthy group were statistically significant (p < 0.05). Nearly 20% of ESBL-producing Enterobacteriaceae isolates were from community-associated intra-abdominal infections and isolates from hospital-associated intra-abdominal infections had more complicated pattern of β-lactamases combinations than isolates from the community from Asia-Pacific region [54].

Conclusion

Our results have shown that patients with gastrointestinal and urinary tract infections harboured more ESBL producing Enterobacteriaceae strains compared with healthy individuals in the community. Although the overall prevalence of ESBL producing bacteria presented in our study is relatively not higher in comparison with other figures reported worldwide. However, the bacterial strains isolated from the patients were more resistant to antibiotics generally used in compacting infections than strains isolated from the healthy volunteers. The high resistance rates to most of the antibiotics tested mandates increased surveillance and molecular characterization of these isolates. Additionally policies to reduce misuse of antibiotics are mandatory, and to stopping investing money in useless antibiotics.

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Bibliography

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