Prevalence of Extended Spectrum Beta-Lactamase Genes among \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} Clinical Isolates

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Extended spectrum beta lactamases (ESBLs) of \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} became a major problem in the whole world. This work aimed to study the prevalence of ESBLs genes (\textit{bla}\textsubscript{TEM}, \textit{bla}\textsubscript{SHV} and \textit{bla}\textsubscript{CTX-M}) in \textit{E. coli} and \textit{K. pneumoniae} clinical isolates recovered from Al Kasr Al-Ainy Hospital, Cairo, Egypt, during the period between 2016 and 2018. One hundred sixty eight clinical isolates were screened for ESBLs production by double disc synergy test (DDST) and combination disc test (CDT). ESBLs genes were detected by PCR using specific primers. Out of 168 isolates, 113 (67.26%) were phenotypically positive ESBLs. Genotypically, only 108 (95.58%) were confirmed as ESBLs producer (61 \textit{E. coli} and 47 \textit{K. pneumoniae} isolates). The percentage of \textit{bla}\textsubscript{CTX-M}, \textit{bla}\textsubscript{SHV} and \textit{bla}\textsubscript{TEM} were 91.7%, 82.4% and 78.7%, respectively. The co-existence of \textit{bla}\textsubscript{TEM}, \textit{bla}\textsubscript{SHV} and \textit{bla}\textsubscript{CTX-M} genes were found in 47.54 and 72.34% for \textit{E. coli} and \textit{K. pneumoniae}, respectively.

Keywords: Enterobacteriaceae, \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, ESBLs.

Introduction

Pathogenic bacteria and their resistance against antibiotics become a worldwide serious problem with challenge for the treatment of infectious diseases. The misuse of antibiotics in medicine, agriculture and veterinary is the main reason for dissemination of resistance genes (Alekhun & Levy, 2007; Lota & Latorre, 2013). Enterobacteriaceae family including more than 70 genera and all bacteria belonging to this family are bacilli, facultative anaerobic bacteria. Usually, their natural host is human and animal, e.g., \textit{Escherichia coli}, \textit{Klebsiella} spp., \textit{Proteus} spp., \textit{Morganella} spp., \textit{Providentia} spp., \textit{Enterobacter} spp. and \textit{Serratia} spp. which cause infections of urinary tract, respiratory tract, blood stream and wounds (Tham, 2012; WHO, 2017).

Beta lactam antibiotics are those containing beta-lactam ring in their chemical structure including: penicillins; cephalosporins and carbapenems (Hamilton-Miller, 1999; Manneznhe et al., 2015). \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} are the dominant extended spectrum \(\beta\)-lactamases (ESBLs) producing organisms isolated globally (Ashrafian et al., 2013; Shakya et al., 2017), that hydrolyze the amid bond in the \(\beta\)-lactam antibiotics and cause resistance to all pencillins, third generation cephalosporins (e.g. ceftazidime, cefotaxime and cefotriaxone) and monobactams (e.g. aztreoname) but not to cephmycins (e.g. cefoxitin and cefotetan) and carbapenems (Bonnet, 2004; CLSI, 2014).

Most of ESBLs are derivatives of the narrow spectrum TEM and SHV type \(\beta\)-lactamases, with one or more amino acid substitutions surrounding their active site. \textit{bla}\textsubscript{CTX-M}, \textit{bla}\textsubscript{SHV} and \textit{bla}\textsubscript{TEM} belong to class A of AmpC classification of ESBLs (Zhao & Hu, 2013). SHV (sulphydryl variant), that originally identified in \textit{K. pneumoniae} (Chaves et al., 2001) and it have greater hydrolytic activity against ceftazidime than other oxyimino-beta-lactams (Tzouvelekis
isolation in 1965, has substrate.

& Bonomo, 1999; Shaikh et al., 2015b). TEM-
jects profiles similar to those of SHV-
(1985, Decious et al., 1990). CTA-M beta-lactamases (ofloxacin-Munich) are derived from K. pneumonia spp. in 1989, (Bauermei et al., 1990), and it is usually topically coded, but in enterobacteria, E. coli and Klebsiella spp., carry the gene of this beta-lactamase on plasmids (Decousser et al., 2001; Poirier et al., 2002; Bush & Jacoby, 2010). Thus, key of this high spread among those bacteria (E. coli and K. pneumoniae) refers to these ESBLs encoded by plasmid-born genes and by which facilities its dissemination (Paterson & Bonomo, 2005; Pitout & Laupland, 2008). Now, these genes are the most prevalent type of ESBLs found in most areas of the world especially, which are commonly identified among enterobacteria, mainly E. coli and K. pneumoniae (Birbrair & Frenette, 2016; Chong et al., 2018).

According to study conducted in 2004 including 28 countries, the rates of ESBL production was 10 and 17% among E. coli and K. pneumoniae isolates with the highest rates being in isolates from Latin America, Middle East, Africa, and Asia and lowest being in Europe and the United States (Rosii et al., 2006; Reinert et al., 2007). Unfortunately, the situation of the Middle East countries was most worrisome where this region seems to be the global ESBL pandemics according to many studies (Al-Agamy et al., 2006; Tawfi et al., 2011; Storberg, 2014). This is due to Many risk factors as poor hygiene and excessive consumption of antibiotics, even without a prescription, as well as non-adherence to the course of treatment, leads to increasing the resistance of bacteria and prevalent of ESBLs especially, in developing countries (Morgan et al., 2011; Ayukbong et al., 2017).

Currently, the recommended therapy for infection caused by ESBL-producing organisms are carbapenems (e.g., imipenem and meropenem, ertapenem, doripenem) where they still the first choice of treatment for serious infections with ESBL-producing E. coli and K. pneumoniae. (Hodiwala et al., 2013; Baral et al., 2018). The aim of this work was to determine the prevalence of ESBLs genes (blaTEM, blaSHV and blaCTX-M) in both E. coli and K. pneumoniae clinical isolates.

Material and Methods

Clinical isolates
One hundred and sixty-eight clinical isolates (85 K. pneumoniae and 83 E. coli) were kindly provided from Al Kasr Al-Ainy hospital during the period between 2016 and 2018. These isolates were recovered from different sources including urine, pus, blood, sputum, semen and stool. Patients’ data (gender and ages) were recorded to obtain possible evidence of correlation between ages, genders and prevalence of ESBLs genes in E. coli and K. pneumoniae.

Bacterial identification
All bacterial isolates were identified by conventional microbiological methods including colony morphology on the MacConkey medium (Oxoid Ltd., Basingstoke, UK), Gram staining and biochemical tests according to Bergey’s manual of systematic bacteriology (Holt & Krieg, 1984), and confirmed by API20E (Bio-Merieux, France) test.

Detection of ESBLs producers
Detection of ESBLs producers was done by phenotypic methods including double disk synergy test (DDST) and combination disk test (CDT) according to (EUCAST, 2013). DDST, carried out by add amoxicillin/clavinic acid (AMC 20/10µg) at the center and around it add cefaziime (CAZ 30µg) or cefotaxime (CTX30µg) at distance 10-15mm, the positive result detected with the inhibition zone is augmented to the direction of AMC disk. CDT, occurred by add disk of CAZ 30µg and CTX 30µg with and without clavinic acid (CV), the positive result detected if the inhibition zone increased by ≥ 5mm larger in clavinic acid combination disk than without.

Antimicrobial susceptibility test
Antimicrobial susceptibility tests were carried out using agar disc diffusion method and according to CLSI guidelines (CLSI, 2013, 2015). The antibiotics used were imipenem (IPM 10µg), meropenem (MEM 10µg), levofloxacin (LEV 5µg), norfloxacin (NOR 10µg), ciprofloxacin (CIP 5µg), ceftaxione (CRO 30µg), cefotaxime (CTX 30µg), ceftazidime (CAZ 30µg), cefoxitin (FOX 30µg); nitrofurantoin (F 300 µg), amikacin (AK 30 µg), piperacillin/tazobactam (TPZ 110µg), Ampicillin (AP 10µg), amoxicillin/clavinic acid (AMC 20/10µg) trimethoprim/sulamethoxazole (SXT 1.25/23.75µg), ceftazidime/clavinic acid (AMC 20/10µg) trimethoprim.
Minimum inhibitory concentrations (MICs) was determined by broth dilution method for the selected isolates that showed positive ESBLs (NCCLS, 2003; EUCAST, 2003), using commercial cefotaxime and ceftazidime powder (GlaxoSmithKline).

Genotypic detection of bla<sub>TEM</sub>, bla<sub>SHV</sub> and bla<sub>CTX-M</sub> by PCR

DNA of E. coli and K. pneumoniae isolates was extracted and purification by Quick-gDNA™ MiniPrep Kit (ZYMO RESEARCH). PCR was carried out using thermal cycler (applied ARKTIK, Germany) where the amplification reaction was as follow: 95°C initial denaturation for 5 min., 30 cycles of (denaturation 95°C for 40 sec., annealing at 53°C for bla<sub>SHV</sub> and at 51°C for bla<sub>TEM</sub> and bla<sub>CTX-M</sub> for 40 sec., extension at 72°C for 40 sec.) and a final extension step at 72°C for 7 min. The PCR products were analyzed by electrophoresis. Selected positive PCR products for bla genes (bla<sub>TEM</sub>, bla<sub>SHV</sub> and bla<sub>CTX-M</sub>) were sequenced by GIS research center, Giza, Egypt. The nucleotide sequences were submitted to GenBank to obtain accession No. The primers used for detection of ESBLs encoding genes bla<sub>TEM</sub> (Schlesinger et al., 2005), blaTEM (Schmiedel et al., 2014) and bla<sub>CTX-M</sub> (Poirel et al., 2001) were listed in Table 1.

Results

Out of 168 bacterial isolates, 113 were phenotypically ESBLs (67.26%) which confirmed by DDST and CDT. According to patients ages and gender among 113 positive ESBLs isolates, the gender was classified to: 52 (46.0%) female patients (including: 42 (37.2%) adult and 10 (8.8%) children); and 61 (54.0%) male patients (including: 57 (50.4%) adult and only 4 (3.5%) children) (Fig. 1).

TABLE 1. Primers for ESBLs genes detection.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Oligonucleotide sequence(5’ - 3’)</th>
<th>Size of amplicons (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHV-F</td>
<td>ATGCGTTATATTGCCCTGTG</td>
<td>747bp</td>
<td>Schlesinger et al. (2005)</td>
</tr>
<tr>
<td>SHV-R</td>
<td>TGGTTGTTATTCCGGGCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM-F</td>
<td>ATGAATTACACATTTGCCG</td>
<td>851bp</td>
<td>Schmiedel et al. (2014)</td>
</tr>
<tr>
<td>TEM-R</td>
<td>TTAATCAGTGAGGCACCTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTX-M-F</td>
<td>CGCTTTGCGATGTGCAG</td>
<td>550bp</td>
<td>Poirel et al. (2001)</td>
</tr>
<tr>
<td>CTX-M-R</td>
<td>ACCGCATATCGTTGTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The isolates were 64 E. coli and 49 K. pneumoniae (confirmed by conventional identification methods and API20E). Figure 2 showed the different sources of positive phenotypic ESBLs clinical isolates where the highest source was urine (53.1%); followed by pus, sputum, blood, and semen with 39.8, 4.4, 1.8 and 0.9 %, respectively.

The antimicrobial susceptibility among ESBLs isolates demonstrated that both E. coli and K. pneumoniae were 100% resistant to CRO and CTX, while for CAZ was 96.89 and 97.96% for E. coli and K. pneumoniae, respectively. Both bacteria were sensitive to carbapenems group including IPM with 100% and MEM with 96.88 and 95.92% for E. coli and K. pneumoniae, respectively (Fig. 3, 4).

MICs values confirmed that all ESBLs producing isolates (E. coli and K. pneumoniae) were highly resistant against cephalosporines (CAZ and CTX). MIC for CTX were ≥512µg/ml for all isolates whereas MIC of CAZ ranged from 128 to > 512µg/ml.

PCR detection for bla genes demonstrated that only 108 (95.92%) isolates (out of 113 ESBLs isolates) were found to have one or more ESBLs genes. Among the positive isolates, the prevalence of bla<sub>SHV</sub>, bla<sub>TEM</sub> and bla<sub>CTX-M</sub> genes were 82.4%, 78.7% and 91.7%, respectively.

PCR was carried out for ESBLs isolates, including 61 E. coli and 47 K. pneumoniae. Single band of the right size was detected for each gene amplicon (Fig. 5). Out of 61 E. coli isolates, the prevalence of bla<sub>SHV</sub>, bla<sub>TEM</sub> and bla<sub>CTX-M</sub> genes were 70.49, 77.05 and 91.8%, respectively. For K. pneumoniae, bla<sub>SHV</sub>, bla<sub>TEM</sub> and bla<sub>CTX-M</sub> genes were 97.87, 80.85 and 91.49%, respectively.

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Fig. 1. Frequency of ESBLs isolates in patients’ gender and ages.

Fig. 2. Different sources of positive phenotypic ESBLs isolates.

Fig. 3. Antibiotics susceptibility among ESBLs E. coli clinical isolates.
Moreover, the coexistence of the 3 genes was detected in 47.54 and 72.34% of *E. coli* and *K. pneumoniae*, respectively. In addition, detection of two genes was found in many isolates, where for *E. coli* isolates were 13.11% (8/61); 19.67% (12/61); 8.2% (5/61) for *bla*<sub>CTX-M</sub> & *bla*<sub>SHV</sub>; *bla*<sub>TEM</sub> & *bla*<sub>TEM</sub>; *bla*<sub>SHV</sub> & *bla*<sub>SHV</sub>, respectively. While for *K. pneumoniae* isolates were 17.02% (8/47); 2.13% (1/47); 6.38% (3/47) for *bla*<sub>CTX-M</sub> & *bla*<sub>SHV</sub> genes; *bla*<sub>CTX-M</sub> & *bla*<sub>TEM</sub>; *bla*<sub>TEM</sub> & *bla*<sub>SHV</sub> (Fig. 6, 7).

Nucleotide sequences of selected ESBLs genes (based on highly purified positive phenotypic and Genotypic ESBLs results) were submitted to *GenBank* under the accession numbers MN096660, MN96662 and MN096664 were for 1K *bla*<sub>TEM</sub>, 98K *bla*<sub>CTX-M</sub>, 107K *bla*<sub>SHV</sub>, respectively, of *K. pneumoniae*. While for *E. coli*, the accession numbers were MN096661, MN096663 and MN096665 for 3E *bla*<sub>TEM</sub>, 97E *bla*<sub>CTX-M</sub> and 69E *bla*<sub>SHV</sub>, respectively.
Discussion

The continued emergence of ESBLs is a serious problem in hospitals as well as, community setting (Shakil et al., 2010). Unfortunately, it is recognized that Egypt has an extremely high rate of ESBL producers (Ahmed et al., 2009; Saied et al., 2011). In this study, out of 168 E. coli and K. pneumoniae clinical isolates, 67.26% were phenotypically positive ESBLs. This percentage is high compared to previous studies in Egypt which was 53.3% (Khater & Sherif, 2014), 42.9% (Storberg, 2014) and 57.8% (Amer et al., 2017). In addition, it was higher than percentage in other countries, Ahmed et al. (2013a) in Sudan and Wadekar et al. (2013) in India that was 59.6%.

ESBLs producers were higher in males (54%) than females (46%) which agreed with the study done by Ben-Ami et al. (2009). While Gibold et al. (2014) found no notable significant difference between the two genders among ESBLs producer’s isolates. In our work, the highest infection was in adult 87.6% while in children was 12.4% which agreed with Gibold et al. (2014) who confirmed higher relative risk in adult patients. Infection in children may be due to chronic medical conditions, prior immunosuppressive therapy, beside similar risk factors to adults (Logan et al., 2014). The highest percent of ESBLs producers was detected in the clinical isolates from urine (53.1%), followed by that from pus, sputum, blood, and semen with
39.8%, 4.4%, 1.8% and 0.9%, respectively. These results agreed with Elsherif & Maamoun (2012) and Shaikh et al. (2015a). In contrast, Ouedraogo et al. (2016) study, recorded that blood cultures was the highest.

Susceptibility test showed that all isolates were resistant to cephalosporins (CAZ, CTX and CRO), while sensitivity was highest to Carbapenems (imipenem; meropenem). This results agreed with Ahmed et al. (2013b) and Ouedraogo et al. (2016) studies who found that ESBL-producing bacteria were resistant to almost all generations of cephalosporins but remained highly susceptible to carbapenems (imipenem and meropenem) which is consistent with the resistance pattern of organisms with ESBLs (Baral et al., 2018). The most effective β-lactam/β-lactamase inhibitor combination against E. coli was TZP (90.62%) followed by AMC (15.63%); whereas against K. pneumoniae were, 71.42 and 18.37% for TZP and AMC, respectively. These results are consistent with other local and global studies done by Ahmed et al. (2013a), Amer et al. (2017). The MIC for E. coli and K. pneumoniae ranged from 128 to >512µg/ml for CAZ; and >512µg/ml for CTX, these results were higher than that demonstrated by Bostanoğlu et al. (2013) but agreed with Peerayeh et al. (2016).

Genotypic characterizations of ESBLs genes showed the prevalence of blaCTX-M genes where it was dominant in almost isolates followed by blaSHV and blaTEM. These findings agree with other studies which reported the prevalence of CTX-M (Abdallah et al., 2015; Ouedraogo et al., 2016; Kpodà et al., 2018). On the other hand, Ahmed et al. (2009) reported that blaTEM was the most frequent β-lactamase encoding gene. This study revealed those ESBLs genes presented 56.48% in E. coli and 43.93% in K. pneumoniae this data agree with Ouedraogo et al. (2016) and Amer et al. (2017) who reported that ESBL producers were more often found in E. coli than K. pneumoniae isolates.

The high percentage of blaSHV (97.87%) in K. pneumoniae indicated the origin of blaSHV is Klebsiella spp (Bush & Fisher, 2011), which accounts for up to 90% resistance in K. pneumoniae (Shaikh et al., 2015b).

The co-productions of ESBLs genes were recorded in most of the isolates either in K. pneumoniae or E. coli. The coexistence of ESBL genes were reported in many studies such as Daef et al. (2009), Salah et al. (2016) and Amer et al. (2017).

Conclusion

The results of our study concluded that the production of ESBLs in Egypt increased in E. coli and K. pneumoniae. Resistance towards 3rd generation cephalosporins especially cefotaxime, ceftazidime confirmed with high MIC for cefotaxime and ceftazidime. The co-existence of ESBLs genes was also recorded. Challenges have to be taken to control outbreaks caused by K. pneumoniae and E. coli.

References


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