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Phenotypic and molecular characterization of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* among Lebanese patients

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Introduction: Antimicrobial resistance is a major public health issue worldwide and became one of the principal international healthcare crises of the 21st century. The production of ESBLs is one of the resistance mechanisms in Enterobacteriaceae, and they are increasingly detected in *Escherichia coli* and *Klebsiella pneumoniae* globally. Therefore, the aim of this study was to determine the phenotypic and molecular characteristics of ESBL-producing *E. coli* and *K. pneumoniae* among Lebanese patients.

Methods: A total of 152 ESBL-producing *E. coli* and *K. pneumoniae* were obtained from Geitaoui Hospital in Beirut between September 2019 and October 2020 from various clinical samples. The phenotype of ESBL producers was confirmed by a double-disc synergy test and antibiotic susceptibility was determined using the disc diffusion method. Genotypically, multiplex PCR was used to detect the ESBL genes (bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV}).

Results: All strains were confirmed to be ESBL producers (121 isolates were *E. coli* and 31 isolates were *K. pneumoniae*). All isolates showed resistance to cefotaxime, cefuroxime, ampicillin and piperacillin. On the other hand, they showed a low susceptibility rate to trimethoprim/sulfamethoxazole and ciprofloxacin. Almost all the isolates were susceptible to ertapenem, imipenem and amikacin. In our study, ESBL genes were detected among 48 (39.67%) *E. coli* isolates and 8 (58.06%) *K. pneumoniae* isolates, and the most prevalent gene was bla_{TEM} (25%), followed by $bla_{CTX\text{-}M}$ (19.08%) and bla_{SHV} (16.45%).

Conclusion: Imipenem and ertapenem are the most effective drugs to treat ESBL producers. However, antibiotic stewardship programs must be implemented immediately to combat antibiotic resistance.

Introduction

Antimicrobial resistance is a global public health concern and it has emerged as one of the most serious international healthcare crises of the 21st century.[1] The most important cause of antimicrobial resistance is the misuse or overuse of antimicrobial agents, which occurs primarily as a result of incorrect diagnosis, inappropriate prescription or poor compliance. Studies have indicated a positive relationship between antibiotic consumption and resistance.[2] One of the resistance mechanisms in Enterobacteriaceae is the production of ESBLs. ESBLs are enzymes that have the ability to hydrolyse the β-lactam ring, resulting in resistance to penicillins and third-generation cephalosporins but not carbapenems and cephamycins.[3] The first report of ESBL production by *Klebsiella pneumoniae* strains was in 1983 and by *Escherichia coli* in 1987.[4,5] There are several types of ESBL; *TEM* and *SHV* were the most common resistance genes but this has changed since 2010, with CTX-M now being the most common in most regions worldwide.[6–8] The first CTX-M β-lactamase was discovered in 1980 and today there are more than 100 variants.[9]

The aim of this study was to determine the phenotypic and genotypic characterization of ESBL-producing *E. coli* and *K. pneumoniae* isolated from Geitaoui Hospital in Beirut, Lebanon.

Methods

Ethics

This study was performed in line with the principles of the Declaration of Helsinki. It was approved by the Lebanese Hospital Geitaoui-University Medical Center (LHG-UMC) Institutional Review Board (IRB), No. 2019-IRB-025. No consent form was needed since the research involved the collection and study of existing diagnostic specimens. The privacy of the patients was guaranteed by labelling the samples with codes rather than patients' names.

Setting and collection of isolates

The study was performed between September 2019 and October 2020 in the biomedical laboratory in Beirut Arab University (BAU). A total of 152 isolates of *E. coli* and *K. pneumoniae* from various clinical specimens were obtained from the microbiology laboratory of LHG-UMC.

Phenotypic testing for ESBL production

The phenotypic test was carried out by modified double-disc synergy on Mueller–Hinton agar where amoxicillin/clavulanic acid (20 μg/10 μg) discs were placed in the centre of the plate and ceftazidime (30 μg), cefotaxime (30 μg), cefuroxime (30 μg), cefoxitin (30 μg), cefepime (30 μg) and aztreonam (30 μg) discs were placed 20 mm away from the amoxicillin/clavulanic acid disc on the same plate. The plate was incubated at 37°C for 24 h and any increase in the zones towards amoxicillin/clavulanic acid was considered as positive for ESBL production.[10]

Antimicrobial susceptibility testing

The Kirby–Bauer disc diffusion technique was used to evaluate the antimicrobial susceptibility of all ESBL producers in accordance with CLSI standards.[11] The following antibiotics were used: ampicillin (10 μg), piperacillin (100 μg), piperacillin/tazobactam (110 μg), amikacin (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), imipenem (10 μg), tetracycline (30ug), ertapenem (10 μg) and trimethoprim/sulfamethoxazole (25 μg). The plates were incubated overnight at 37°C and the findings were classified as susceptible, intermediate or resistant.

Characterization of genes encoding ESBLs

For molecular characterization, a multiplex PCR was done using primers for the three genes SHV, TEM and CTX-M listed in Table 1.[12]

PCR amplification

PCR amplification reaction was performed in a volume of 20 μL containing: 10 μL master mix reaction buffer (containing 0.05 μL Taq polymerase, 4 mM $MgCl_2$ and 0.4 mM dNTPs) and a 10 μL mix containing DNA (5 μL), primer mix [forward (0.5 μL) and reverse (0.5 μL)] for the three genes and nuclease-free water (2 μL). Reactions were performed in a DNA thermal cycler (BIOER) under the following conditions: initial denaturation at 95°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 62°C for 90 s, extension at 72°C for 60 s and final extension at 72°C for 10 min.[12] After PCR amplification, 12 μL of each reaction was separated by electrophoresis in 1.5% agarose gel for 45 min at 100 V in 1× Tris-borate-EDTA (TBE) buffer. DNA was stained with ethidium bromide (1 μg/mL) and the bands were detected using a UV transilluminator.

Table 1. Sequences of the primers used in this study to amplify regions of TEM, SHV and CTX-M, and sizes of the PCR amplicons[12]

Target (s)	Primer sequence (5′ to 3′)	Size (bp)
TEM	TEM-F: CGC CGC ATA CAC TAT TCT CAG AAT GA TEM-R: ACG CTC ACC GGC TCC AGA TTT AT	445
SHV	SHV-F: CTT TAT CGG CCC TCA CTC AA SHV-R: AGG TGC TCA TCA TGG GAA AG	237
CTXM	CTXM-F: ATG TGC AGY ACC AGT AAR GTK ATG GC CTXM-R: TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593

Results

Of the total 152 isolates, 121 (79.61%) *E. coli* and 31 (20.39%) *K. pneumoniae* were phenotypically confirmed to be ESBL producers. The majority of the isolates (127; 83.5%) were isolated from urine samples. Other specimen sources were sterile body fluids (7; 4.61%), wound swabs (7; 4.61%), sputum (6; 3.95%), blood (3; 1.97%) and pus (2; 1.32%). Seventy-five isolates were from males and 77 from females.

Phenotypic characteristics

The results revealed that all isolates were resistant to cefuroxime, cefotaxime, ampicillin and piperacillin. The susceptibility rates of isolates to other antibiotics were 7% to aztreonam, 8% to cefepime, 24% to ceftazidime, 36% to trimethoprim/sulfamethoxazole, 38% to ciprofloxacin, 41% to tetracycline, 51% to amoxicillin/clavulanic acid, 70% to gentamicin, 70% to tazobactam/piperacillin and 89% to amikacin. All isolates were susceptible to cefoxitin, ertapenem and imipenem.

Molecular characteristics

Molecular methods showed that 66 (43.42%) of the isolates harboured at least one of the ESBL- related genes. Eighteen (58.06%) of the *K. pneumoniae* isolates harboured at least one gene and 48 (39.67%) of the *E. coli* isolates harboured at least one gene. The bla_{TEM} gene was the most prevalent gene in *E. coli* isolates while the bla_{SHV} gene was the predominant gene in *K. pneumoniae*.

Almost half of the positive isolates harboured two or more ESBL genes. The number of strains harbouring all three genes was 4 (2.63%); the number harbouring both $bla_{CTX\text{-}M}$ and bla_{TEM} was 15 (9.87%); the number harbouring both bla_{TEM} and bla_{SHV} was 10 (6.58%); and the number harbouring both $bla_{CTX\text{-}M}$ and bla_{SHV} was 5 (3.29%).

Discussion

The emergence of ESBL has been identified as a major global concern. In Lebanon, the prevalence of ESBL production in *Klebsiella* species and *E. coli* for hospitalized patients in 2013 was 29.2% and 32.3% respectively, while in 2016, the percentage susceptibility of *Klebsiella* and *E. coli* strains to third-generation cephalosporins for hospitalized patients was 63% and 58%, respectively.[13,14]

In our study, we focused on determining the phenotypic and molecular characterization of ESBL-producing *E. coli* and *K. pneumoniae* isolated from Geitaoui Hospital in Beirut, Lebanon over a period of 1 year. In this study, the majority of patients (83.5%) had urinary tract infection, with *E. coli* being the most prevalent pathogen (79.61%), followed by *K. pneumoniae* (20.39%). Our findings are consistent with those of a previous study conducted in Lebanon, which showed that *E. coli* has the highest incidence in urinary tract infection followed by *K. pneumoniae*.[15]

Comparing our antibiotic susceptibility results with a previous study in Lebanon, we observed a slight decrease in susceptibility.[10] Cefepime showed high differences between results, where

the susceptibility percentage dropped from 35.6% to 8%. Even gentamicin dropped from 91.5% to 70%. These results showed that the resistance among ESBL-producing *E. coli* and *K. pneumoniae* is increasing due to many factors mainly the overuse of these agents.

The high rate of resistance to aztreonam, cefepime, trimethoprim/sulfamethoxazole and ciprofloxacin showed the increasing prevalence of resistance rates in Lebanon. However, most of the isolates (89%) showed high susceptibility to amikacin. These results are in accordance with other studies conducted in Pakistan and Canada.[16,17] All our isolates were susceptible to ertapenem and imipenem. This finding is similar to the work done in Lebanon by Ghaddar and colleagues,[10] which showed high susceptibility to imipenem (93.2%).

Concerning the molecular characteristics, most studies done in Lebanon showed that the most prevalent gene in ESBL was $bla_{\text{CTX-M}}$.[10,18] In Iraq, the most prevalent gene was bla_{TEM}, followed by $bla_{\text{CTX-M}}$.[19] However, this was not the case in our study, which showed that the most prevalent genes in ESBL-producing *E. coli* and *K. pneumoniae* were bla_{TEM} and bla_{SHV}, respectively. These findings were compatible with a study done in India.[20] Our study clearly showed the change in the gene pool in the Lebanese Enterobacteriaceae isolates. Several other studies conducted worldwide revealed different results, which means that the prevalence of ESBL genes may differ by geographical location. Moreover, in our study, 86 (56.58%) of the phenotypically positive ESBL strains lacked CTX, SHV and TEM genes. So, we assume that other ESBL-encoding genes exist in the Lebanese population studied.

Conclusions

In conclusion, Lebanon showed an increase in resistance to almost all antimicrobial drugs, except carbapenems, which were the most effective against ESBL-producing bacteria. This study confirmed the urgent need to implement antibiotic stewardship, which is a program that promotes the proper prescription of antibiotics by physicians and use by patients.

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Transparency declarations

Authors declare no conflict of interest.

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