Original Article

Molecular detection of plasmid-derived AmpC β-lactamase among clinical strains of *Enterobacteriaceae* in Bahrain

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Abstract:

BACKGROUND: *Enterobacteriaceae* with AmpC β-lactamase are multidrug-resistant organisms and represent a significant challenge to patient care. This study aims to determine the prevalence of plasmid-derived AmpC β-lactamase among extended spectrum β-lactamases (ESBL)-producing *Enterobacteriaceae* strains in Bahrain.

METHODS: It was a cross-sectional study. A total of 185 ESBL-producing *Enterobacteriaceae* isolates were recovered from clinically significant specimens from January 2018 to December 2019. The samples underwent initial screen for cefoxitin resistance by disc diffusion test and subsequent phenotypic confirmation of AmpC production with phenyl boronic acid assays as well as genotypic analysis by multiplex polymerase chain reactions for AmpC subtypes. Drug-resistant features of these clinical isolates were also examined.

RESULTS: Twenty-nine ESBL-producing *Enterobacteriaceae* isolates were cefoxitin resistant. Phenotypic and genotypic analyses confirmed that 8 and 12 cefoxitin-resistant isolates are AmpC positive, respectively. These AmpC producers are multidrug resistant, and *Escherichia coli* is the dominant strain among them.

CONCLUSIONS: Plasmid-mediated spread of AmpC is present in clinically relevant *Enterobacteriaceae* species in Bahrain. Rational antimicrobial therapy against these multidrug-resistant organisms and continued surveillance of antimicrobial resistance mechanisms among the clinical isolates are recommended for optimal patient care.

Keywords:

AmpC genotypes, antimicrobial resistance, cefoxitin resistance, *Enterobacteriaceae,* multiplex polymerase chain reaction

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Enterobacteriaceae represents a large family of Gram-negative bacteria that are rod-shaped facultative anaerobes, with 51 genera and 238 species. These microbes could be distinguished from other rod-shaped Gram-negative bacteria based on morphological and functional features as well as the presence of Enterobacterial common antigen.[1] Nevertheless, genetic analyses have replaced these types of phenotypic characterization as the standard identification method for *Enterobacteriaceae* strains.[1] These bacteria are ubiquitous in nature and are able to take on a variety of ecological environments, ranging from terrestrial and aquatic niches to animals and insects. While *Enterobacteriaceae* could be considered as biocontrol agents in agriculture, health care, and environmental management, many species, such as *Escherichia coli, Klebsiella pneumoniae,* and *Proteus mirabilis,* are known as opportunistic

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pathogens, causing many types of infections in humans.[1,2]

Traditional approaches to treating *Enterobacteriaceae* infections involve a single class of antibiotics, such as aminoglycoside, fluoroquinolone, cephalosporin, or carbapenem. However, repeated exposure to these antibiotics leads to drug-resistant *Enterobacteriaceae*. In recent years, members of this family have become increasingly resistant to antimicrobial therapies, creating a therapeutic conundrum in hospital and community settings.[2] In this regard, β-lactam resistance is commonly observed among the *Enterobacteriaceae* strains. Some *Enterobacteriaceae* are capable of producing enzymes called β-lactamases, which inactivates β-lactam antibiotics by cleaving its central ring structure that is responsible for the antimicrobial activity of these agents.[3]

β-lactamases that have been encountered in *Enterobacteriaceae* species include carbapenemases, oxacillinases, metallo-β-lactamases (MBL), extended spectrum β-lactamases (ESBL), and AmpC β-lactamases.[3-5] In clinical settings, the most commonly detected β-lactamases are AmpC and ESBLs.[2] Notably, AmpC β-lactamases confer resistance to β-lactam/β-lactamase inhibitor combinations, narrow as well as broad-spectrum cephalosporins, and aztreonam.[6] There are two main types of AmpC in *Enterobacteriaceae* species: chromosomal and plasmid derived.[7,8] The former is often generated by chromosomal deregulation, whereas the latter comes from gene transfer from plasmids. Subtypes of plasmid-derived β-lactamases have been named according to their resistance to cefoxitin (FOX), cephamycins (CMY), and moxalactam (MOX) or latamoxef (LAT). In addition, these β-lactamases could be classified by the species origin such as CIT (*Citrobacter freundii*) and EBC (*Enterobacter cloacae*); their genetic features such as Ambler class C (ACC) and AmpC type (ACT); their discovery sites such as the Dhahran Hospital in Saudi Arabia (DHA) and Miriam Hospital in Providence (MIR-1); or the name of the subject in which the species was discovered (BIL-1, patient Bilal).[7,9] Among these, ACC, FOX, MOX, DHA, CMY, CIT, and EBC genotypes are most commonly reported.[8]

In Europe and the United States, the prevalence of AmpC genes and antibiotic resistance due to AmpC enzymes have been reported.[10,11] Similarly, the AmpC-producing *Enterobacteriaceae* isolates have also been documented in Saudi Arabia.[12,13] Furthermore, many studies have reported an alarming rise of ESBL and AmpC co-producer strains, causing significant challenges for the management of infections as these *Enterobacteriaceae* bacteria are multidrug-resistant organism.[14,15] Furthermore, the low level of AmpC expression in *E. coli* and absence of chromosomal AmpC in *K. pneumoniae* highlight the importance of plasmid-mediated AmpC transfer in these bacteria.[15]

Accurate detection of AmpC is not only critical for proper health management of patients suffering from *Enterobacteriaceae* infections but also useful for epidemiological analyses of the geographical distribution of AmpC genes. To date, no clinical studies on the prevalence of AmpC β-lactamases have been conducted in Bahrain. There are also currently no Clinical and Laboratory Standards Institute guidelines (CLSI) for AmpC detection in Gram-negative clinical isolates, which often results in diagnostic errors in phenotypic tests.[16] Furthermore, chromosomal and plasmid-derived AmpC genes cannot be differentiated by phenotypic tests, prompting the urgent need for a gold standard genotypic detection of pAmpC types.[14] Therefore, the goal of this study is to report a methodological standard for plasmid-derived AmpC detection as well as to examine the frequency of these plasmid-derived-AmpC positive organisms among ESBL-producing clinical strains of *Enterobacteriaceae* in the Kingdom of Bahrain.

Methods

Bacterial isolate collection and identification

This was a cross-sectional study. The prospective analysis was based on clinical laboratory surveillance from January 2018 to December 2019 at the Salmanyia Medical Complex in the Kingdom of Bahrain. The dataset includes 185 ESBL-producing *Enterobacteriaceae* strains (123 *E. coli* samples, 56 *K. pneumoniae* samples, and 6 *P. mirabilis* samples). All the bacterial isolates were identified using Matrix-assisted laser desorption ionization time-of-flight mass spectrometry , Bruker, Bremen, Germany. (MALDI-TOF) (Bruker) in the clinical microbiology lab at the Salmanyia Medical Complex. The study received institutional review board approval (number E014-PI-11/16) from the ethics committee of the university.

AmpC β-lactamase screening

ESBL-producing isolates were analyzed for AmpC production by the Kirby Bauer disc diffusion method with 30 μg cefoxitin as previously described.[17] Bacterial isolates that yielded a zone diameter of <18 mm were considered as positive for ESBL production. Furthermore, an inhibitor-based method using boronic acid was employed as the phenotypic confirmatory assay [Figure 1a].[18] Briefly, the test discs were prepared with 30 μg cefoxitin and 20 μL of the stock boronic acid solution, which was prepared by dissolving 120 mg boronic acid in 3 mL dimethyl sulfoxide and subsequently diluting this solution with 3 mL of sterile distilled water. A set of two test discs with and without 400 μg phenyl boronic acid

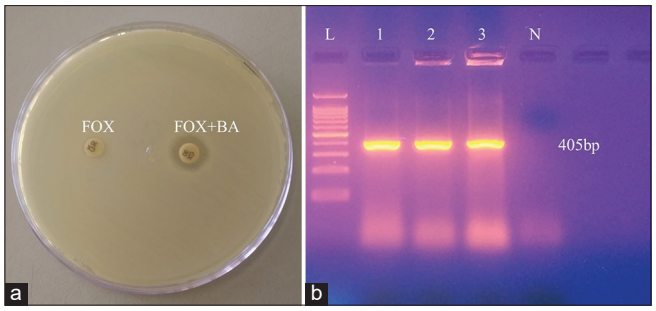

Figure 1: Phenotypic and molecular analyses of bacterial isolates. (a) Representative result of the boronic-acid based assay to identify AmpC-producing isolates with FOX (cefoxitin alone) and FOX + BA (cefoxitin + boronic acid) test discs. (b) Representative gel electrophoresis result of polymerase chain reaction products derived from bacterial isolates with the presence of 405 base-pair (bp) products (DHA gene) in lanes 1–3. Lane N represents water negative control and lane L contains the molecular ladder

were placed at a distance of 30 mm on Mueller–Hinton agar plate and inoculated with bacterial isolates at 37°C overnight. An increase in zone diameter of >5 mm in the presence of cefoxitin and phenyl boronic acid in comparison with cefoxitin alone was considered to be positive for AmpC β-lactamase production.

Molecular characterization of AmpC resistance

Samples for genotypic confirmation were obtained from blood; urine; pus; pleural fluid; and suprapubic, wound, rectal, and perianal swabs. Multiplex polymerase chain reactions (PCR) was used to detect the most common plasmid-mediated AmpC genes as shown in Table 1.[19] For PCR assays, 2-μL cDNA was added to 23-μL master mixture of PCR reagents. The reaction was programmed with initial denaturation step at 94°C for 3 min, followed by 25 cycles of DNA denaturation at 94°C for 30 s primer annealing at 64°C for 30 s, primer extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. 15-μL of PCR products was analyzed by gel electrophoresis with 2% agarose and visualized by ultraviolet transillumination [Figure 1b]. A 100 base-pair DNA ladder was used as the size reference and PCR mix with distilled water was used as the negative control.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 20 (IBM, Armonk, NY, USA) was used to obtain descriptive data. The significance was measured by Chi-square test.

Results

Escherichia coli represents the dominant AmpC-β-lactamase-positive bacterial strain in extended spectrum β-lactamases-producing clinical isolates

Among 185 ESBL-producing *Enterobacteriaceae* isolates, cefoxitin resistance was documented in 15.3% (*n* = 29) of the samples by the disc diffusion test. AmpC β-lactamase production was phenotypically confirmed by inhibitor-based method in 4.3% (*n* = 8) and genotypically confirmed by PCR analysis in 6.4% (*n* = 12) of all clinical isolates. Genotypic AmpC-β-lactamase producers were obtained from urine (*n* = 9), wound (*n* = 2), and perianal (*n* = 1) swabs, which could be due to patients' previous antimicrobial exposure or health care-related acquisition. They could further be assigned to CIT (*n* = 9) and DHA subtype (*n* = 3) [Table 2]. Notably, 73% of cefoxitin-resistant isolates contain *E. coli* strain (*n* = 21), whereas the remaining samples consist of *K. pneumoniae* (*n* = 5) and *P. mirabilis* strains (*n* = 3) [Figure 2a]. *E. coli* strain also represent 87% and 91% of phenotypic (*n* = 7) and genotypic (*n* = 11) AmpC β-lactamase producers, respectively [Figure 2b and c].

Table 1: Primers used for the characterization of AmpC β-lactamases

Primer	Sequence (5′ to 3′)	Expected amplicon size (bp)
MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
MOXMR	CAC ATT GAC ATA GGT GTG GTG C	
CITMF	TGG CCA GAA CTG ACA GGC AAA	462
CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
DHAMR	CCG TAC GCA TAC TGG CTT TGC	
ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
ACCMR	TTC GCC GCA ATC ATC CCT AGC	
EBCMF	TCG GTA AAG CCG ATG TTG CGG	302
EBCMR	CTT CCA CTG CGG CTG CCA GTT	
FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
FOXMR	CAA AGC GCG TAA CCG GAT TGG	

Multi-drug resistance is a cardinal feature of cefoxitin-resistant and genotypically AmpC-positive isolates

Drug resistance analysis was carried out for several types of antibiotics, including cefuroxime, ciprofloxacin, nitrofurantoin, meropenem, gentamicin, fosfomycin, and tigecycline, as detailed in Tables 3 and 4. All the clinical isolates are resistant to cefuroxime. Interestingly, the frequencies of cefoxitin-resistant and genotypically AmpC-positive isolates that are resistant to ciprofloxacin, and fosfomycin, were notably higher than that of AmpC-negative isolates [Tables 3 and 4]. Furthermore, while multiple drug resistance was observed in both genotypically AmpC-positive isolates and AmpC-negative isolates, the frequency of genotypically AmpC-positive isolates (25%) that are resistant to more than four antibiotics was higher than that of AmpC-negative isolates (19%) [Figure 3]. Collectively, these findings were consistent with the notion that the development of multidrug resistance is associated with AmpC β-lactamase synthesis in ESBL-producing *Enterobacteriaceae*.

Discussion

Resistance to β-lactam antibiotics in ESBL-producing *Enterobacteriaceae* isolated from clinical samples, notably *E. coli*, *K. pneumoniae*, *P. mirabilis*, and *Enterobacter aerogenes*, has been increasing worldwide. Notably, ESBL phenotypes are complex due to the co-production of AmpC.[20] Even though there are currently no CLSI guidelines for AmpC detection, reduced cefoxitin susceptibility has been used as an indicator for AmpC producers in ESBL-producing clinical isolates. In the present study, 15.3% of all ESBL producers are cefoxitin resistant. The low frequency of cefoxitin-resistant ESBLs in Bahrain is in stark contrast with those identified in other countries. For instance, studies in Iran revealed that the prevalence of cefoxitin-resistant strains is 40%–68.4% of all ESBL strains.[15,21] A high prevalence of cefoxitin-resistant ESBLs has also been documented in Portugal.[22] This reduced susceptibility to cefoxitin among ESBL producers serves as an important alarm for the spread of plasmid-derived AmpC genes in Bahrain.

Similarly, the prevalence of ESBL and AmpC co-producers has been reported with great heterogeneity in the world. While this study revealed that 6.4% of ESBL-producing strains are genotypically AmpC positive, a study from Turkey reported the prevalence of these strains to be 38% of all clinical isolates.[23] In India, Handa *et al*. reported that these strains exist at a markedly higher frequency of 84.62%.[24] However, other reports in India showed a range of prevalence from 8.39 to 33%.[20,25-27] This study also showed that 8.9% of *E. coli* and 16.6% of *P. mirabilis* isolates exhibited co-production of ESBL and AmpC, whereas none of the *K. pneumoniae* samples harbored AmpC gene. In contrast, Kaur *et al*. reported the presence of ESBL and AmpC co-producers in 12.1% of *E. coli*, 13.8% of *K. pneumoniae*, and 1% of *P. mirabilis* samples.[20] In addition, Manoharan *et al*. reported frequencies of AmpC in *E. coli* and *K. pneumoniae* at 43.7% and 16.6%, respectively.[16] These diverse prevalence rates might result from geographical differences in antimicrobial susceptibility as well as differences in phenotypic or genotypic detection methods. More importantly, the frequency of clinical isolates that are resistant to more than four antibiotics is higher in the co-producers of ESBL and AmpC than nonproducers in this study. Therefore, along with the differences in previous studies about the diversity of drug resistant ESBL and AmpC co-producers, these findings are of particular epidemiological concern about the potential of antibiotic misuse in different region of the world in aiding the emergence and spread of multidrug-resistant pathogenic bacteria.

With regard to diagnosis, the knowledge about the molecular subtypes and the prevalence of plasmid-derived AmpC in different geographical areas is critically important for proper consideration of antimicrobial therapy and efficient infection control.

Table 2: Distribution of AmpC-related genes among AmpC-producing isolates

Strain	ACC	FOX	MOX	DHA	CIT	EBC
Escherichia coli	0	0	0	3	8	0
Klebsiella pneumoniae	0	0	0	0	0	0
Proteus mirabilis	0	0	0	0	1	0

ACC=Ambler Class C, FOX=Cefoxitin, MOX=Moxalactam, DHA=Dhahran Hospital in Saudi Arabia, CIT=*Citrobacter freundii*, EBC=*Enterobacter cloacae*

Table 3: Antibiotic resistance pattern of cefoxitin-resistant and AmpC-negative strains

Antibiotic type	Cefoxitin-resistant strains (*n*=29), *n* (%)	AmpC-negative strains (*n*=173), *n* (%)	95% CI	*P*
Cefuroxime	29 (100)	173 (100)	NA	NA
Ciprofloxacin	20 (68.9)	44 (25.4)	0.255- 0.616	<0.0005
Nitrofurantoin	6 (20.6)	69 (39.8)	−0.356- −0.027	0.048
Meropenem	4 (13.7)	9 (5.2)	−0.044- 0.216	0.081
Gentamicin	9 (31.0)	73 (42.1)	−0.295- 0.072	0.257
Fosfomycin	13 (44.8)	35 (20.2)	0.055- 0.437	0.004
Tigecycline	11 (37.9)	27 (15.6)	0.039- 0.408	0.004

*P<0.05 is considered statistically significant. CI=Confidence interval, NA=Not available

Table 4: Antibiotic resistance pattern of AmpC-producing and AmpC-negative strains

Antibiotic type	AmpC-producing strains (*n*=12), *n* (%)	AmpC-negative strains (*n*=173), *n* (%)	95% CI	*P*
Cefuroxime	12 (100)	173 (100)	NA	NA
Ciprofloxacin	8 (66.6)	44 (25.4)	0.138- 0.687	0.002
Nitrofurantoin	4 (33.3)	69 (39.8)	−0.342- 0.211	0.653
Meropenem	1 (8.3)	9 (5.2)	−0.129- 0.191	0.643
Gentamicin	3 (25)	73 (42.1)	−0.428- 0.084	0.242
Fosfomycin	7 (58.3)	35 (20.2)	0.096- 0.666	0.002
Tigecycline	2 (16.6)	27 (15.6)	−0.207- 0.228	0.922

*P<0.05 is considered statistically significant. CI=Confidence interval, NA=Not available

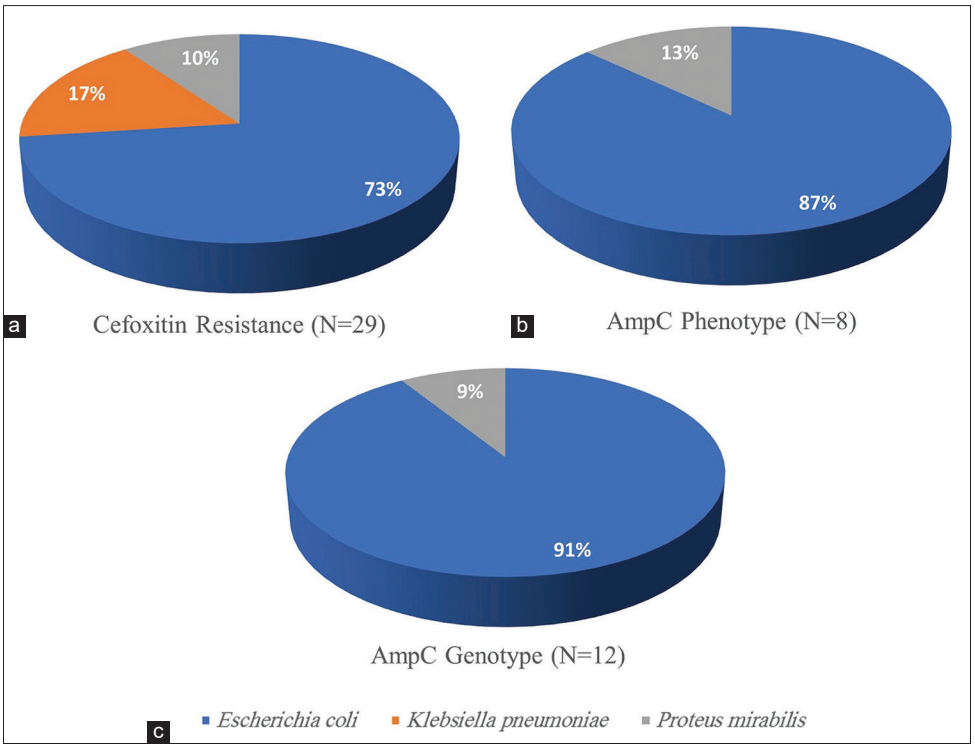

Figure 2: Phenotypic and genotypic characterization of extended spectrum β-lactamases-producing isolates. Frequencies of different bacterial strains among extended spectrum β-lactamases-producing isolates that were identified as cefoxitin resistance (a), phenotypic AmpC-β-lactamase producers (b) and genotypic AmpC-β-lactamase producers (c)

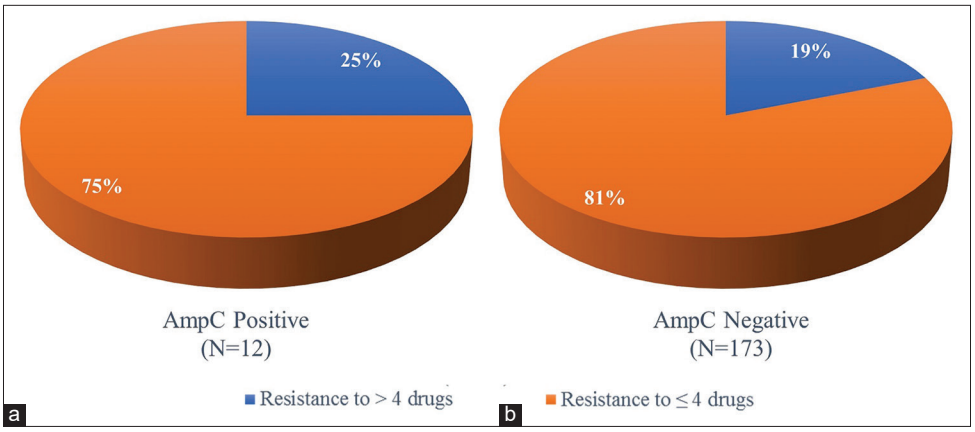

Figure 3: Prevalence of multi-drug-resistant extended spectrum β-lactamases-producing isolates (a). Frequencies of clinical isolates that (b) are resistant to more than four antibiotics in genotypic AmpC β-lactamase producers (AmpC positive) and nonproducers (AmpC negative)

The present study revealed that the prevalence of ESBL and AmpC co-producers is 4.3% by disc diffusion method and 6.4% by PCR analysis. Discrepancy between prevalence results yielded by genotypic and phenotypic confirmation assays for AmpC has also been reported by Zorgani *et al*.[28] The lack of specificity of phenotypic method of detection of AmpC might be accountable for this observation. Therefore, while phenotypic tests are commonly applied in the clinical laboratories, molecular methods should be considered the gold standard for β-lactamase detection, identification, and for epidemiological knowledge.[22,29] Furthermore, these results suggest that while ESBL producers might be more prevalent and spread much more easily than AmpC, careful monitoring of the latter needs to be emphasized due to the aforementioned issues in AmpC confirmation assays.

In the present study, 10.3% and 31% of cefoxitin-resistant isolates harbored the DHA and CIT gene, respectively. Studies in different areas of the world have revealed geographical diversity in the molecular subtypes of AmpC genes. In this regard, CIT subtype of AmpC has been widely detected in the United States and Canada

in recent years.[30] Woodford *et al*. also described the predominance of CIT-positive, AmpC-producing *E. coli*, in the UK and Ireland.[31] In contrast to these studies, Wassef *et al*. reported MOX and FOX families as the most prevalent AmpC subtypes in Egypt, followed by EBC and CIT subtypes.[32] Adding to the geographical complexity of AmpC strains, studies in North Africa and Australia have reported CMY, DHA, and EBC as the most frequent subtypes of AmpC producers.[28,33] In India, Manoharan *et al*. reported that CIT-FOX-like and EBC-like enzymes were common in *E. coli* (43.7%) and *K. pneumoniae* (16.6%), while Govindaswamy *et al*. showed that FOX gene was the predominant AmpC subtype (21.9%).[16,34] Lastly, in the Gulf states, minimal data exist with regard to AmpC subtypes with the documented presence of CMY-4/CMY-6 in the United Arab Emirates and Kuwait and DHA1/CMY-2 in Saudi Arabia.[12] Collectively, these discrepancies are of particular scientific interest about the evolution of AmpC subtypes worldwide.

In congruence with previous reports in India, Pakistan, Korea, and Spain, the majority of the strains with AmpC genotype are generally multidrug resistant.[35-37] The present study also observed that all ESBL producers, regardless of their AmpC status, are resistant to cefuroxime, of which resistance in strains with AmpC genotype has been reported at a high rate (>94%).[13,38] These findings are also consistent with resistance mechanisms against cerufoxime, which involves hydrolysis by ESBL and AmpC enzymes. Other antibiotics that AmpC-positive strains in this study are resistant to include gentamicin (42.1%), nitrofurantoin (39.8%), ciprofloxacin (25.4%), tigecycline (15.6%), and meropenem (5.2%). Notably, least resistance is observed with meropenem, which belongs to the antibiotic class of carbapenems – the first-line choice for treating ESBL and AmpC β-lactamase-associated infections.[13] While these results are similar to those presented in a study by Bindayna and Murtadha,[39] increasing rate of resistance to meropenem (28.6%) has also been noted by studies by Ibrahim *et al*. and Marie *et al*.[13,40] Mechanistically, overuse of carbapenems can stimulate the selection of β-lactamases that are capable of hydrolyzing carbapenems and outer membrane protein mutations. Therefore, in order to avoid the emergence of carbapenem-resistant strains, efforts should be made to limit the use of this class of antibiotics if an equally effectual alternate drug exists.[41] The limitation in our study was that we did not perform molecular techniques to detect the various ESBL genes in our isolates. The other limitation is that we did not look for the other possible resistance mechanisms such as MBL and efflux pump.

Conclusions

Our findings are the first to document the spread of plasmid-derived AmpC genes in the Kingdom of Bahrain. They also highlight the predominant existence of ESBL and AmpC co-producers in multidrug-resistant *E. coli*. These findings suggest that rational antimicrobial therapy and continued meticulous surveillance are critical to effectively curb the dissemination of these clinical strains.

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Conflicts of interest

There are no conflicts of interest.

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