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Plasmid mediated extended spectrum beta-lactamase producing strains of enterobacteriaceae isolated from diabetic foot infections in Egypt

Kamel, Noha. A [1], Aboshanab, Khaled M.[2], Abouelwafa, Mohammad M.[2], El-tayeb, Wafaa N.[1]

1 Department of Microbiology, Faculty of Pharmacy, Misr International University (MIU), Cairo, Egypt.

2 Department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Organization of African Unity St., POB: 11566, Abbassia, Cairo, Egypt.

Correspondence:

aboshanab2003@yahoo.com

Abstract

Background: Extended spectrum beta-lactamases (ESBLs) are heterogeneous group of plasmid mediated β-lactamases enzymes that confer resistant to oxyimino cephalosporins, monobactam (aztreonam) and usually show co-resistance to other classes of antibiotics. The present was done to identify and characterize the most common plasmid-mediated ESBL genes associated with diabetic foot infections in Egypt.

Methods and findings: A total of 135 Gram negative bacterial isolates were recovered from 91 diabetic foot ulcers specimens of patients attending governmental hospitals and private clinics in Cairo, Egypt. The antibiogram analysis revealed a remarkable high resistance pattern towards different classes of the tested antimicrobial agents. Based on CLSI, a total of 114 isolates out of 135 were considered potential ESBLs producer by using disc diffusion and broth microdilution screening assays followed by double disc synergy test (DDST). 58 out of 114 isolates were ESBLs producers and almost 14% (8 out of 58), were plasmid-mediated as determined by plasmid extraction and transformation experiments. The majority of the tested plasmids (6 out of 8) carried 2 or more genes on the same plasmid. The most common combination was bla_{CTX-M} and bla_{TEM} (n=3/8; 37.5%), followed by bla_{SHV} and bla_{CTX-M}(n=2/8; 25%) and bla_{CTX-M}+ bla_{TEM}+ bla_{SHV} (n=1/8;12.5%). One *E.coli* isolate harbored a plasmid (pECDF16) coding for the three ESBLs genes. The final nucleotide sequences of bla_{TEM1} and bla_{SHV-8} were submitted to the GenBank database under accessions coder JX976326, and JX976327, respectively.

Conclusion: High prevalence of plasmid-mediated ESBLs was detected among DFIs in Egypt. Therefore, new guidelines should be undertaken in Egypt to limit or prevent the misuse and abuse of antimicrobial agents.

Key words: ESBLs, plasmid-mediated ESBLs, Diabetic foot infections, Enterobacteriaceae

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Introduction

By year 2030, Egypt will be from the top countries that suffers from Diabetes mellitus [1]. Being diabetic, places patient at a high burden to develop diabetes chronic complications. Diabetic foot ulcers are considered to be one of the most common complications of diabetes, that are usually vulnerable to infections that spreads rapidly, leading to devastating tissue destruction and increases risk of leg amputation [2]. Most diabetic foot infections (DFI) are polymicrobial (where two or more bacteria are recovered) from the same specimen. In addition to Gram positive staphylococci, Gram negative rods member of Enterobacteriaceae and *Pseudomonas aeruginosa* may be recovered [3]. One of the major concern is

the increasing incidence of multidrug-resistant Gram negative organisms, particularly extended spectrum beta lactamases producers (ESBLs) among DFI [4].

ESBLs are Class A β-lactamases that have the ability to hydrolyze oxyimino cephalosporins and monobactams. However, these enzymes cannot hydrolyze cephamycins or carbapenems [5] and they are *in vitro* inhibited by clavulanate [6]. The majority of the ESBLs are plasmid mediated (mega plasmids) that can has resistant determinants to quinolones and aminoglycosides in addition to, β-lactam hence limiting therapeutic options [7, 8]. Moreover, being plasmid mediated will facilitate the propagation of these determinants among different bacterial species via horizontal transfer.

The vast majority of the clinically important ESBLs belong mainly to TEM (called after Greek girl "Temoniera"), SHV (refers to sulfhydryl variable) and CTX (called after conferred resistance to cefotaxime) genotypes [9]. These ESBLs have evolved as a result of substitutions in key amino acids of parent TEM-1/2, SHV-1 enzymes [6] and incorporation of pre-existing ESBL genes on chromosome of *Kluyvera* spp. onto mobile plasmid, respectively [10]. Those point mutations within ESBLs will lead to huge diversity within ESBL genotype. Subsequently, molecular characterization of all suspected Gram negative ESBL producers (phenotypically detected) is of immense important to detect the spread and to identify the exact variant within resistant genes recovered from DFIs. Therefore, this study was conducted to study prevalence of plasmid-mediated ESBLs among DFI in Egypt and determine correlation between phenotypic and genotypic data of antibiotic resistance among relevant pathogens.

Methods

Microorganisms, and phenotypic detection of ESBLs

A total of 206 aerobic/facultative anaerobes clinical bacterial isolates were recovered from 91 diabetic foot ulcers (DFUs). The bacterial isolates were identified via conventional methods (direct film, macroscopical characters and standard biochemical tests). Gram negative isolates were chosen to further screening for potential ESBLs producers using disc diffusion and micro broth dilution method [11, 12]. For more confirmations of ESBLs detection, the phenotypic double disc synergy test (DDST) was carried out on potential ESBLs producer [13].

Genotypic detection of plasmid-mediated ESBLs

For the detection of plasmid mediated ESBLs among our isolates, the phenotypically confirmed ESBLs producer by DDST, were subjected to plasmid extraction by alkaline lysis as described by Birnboim and Doly, 1979 [14]. In a trial to prove the association of these plasmids with ESBLs, genetic transfer of the extracted plasmids via transformation was carried out using competent *E. coli* DH5α and *E. coli* JM109 (Novagen, Darmstadt, Germany). These competent cells were prepared and transformed according to the modified Hanahan method [15] and Sambrook and Russell [16]. PCR technique was used to detectand amplify the most common ESBLs genes via using universal primers for bla_{TEM} [17], bla_{SHV} [17], bla_{CTX-M} [18] and plasmid DNA as templates. PCR amplification program was computerized using pDRAW32 (http://www.acaclone.com).

DNA sequencing of PCR products

Prior to sending PCR products to sequencing, a PCR cleanup step was performed using GeneJET Purification Kit. The purified PCR products were sent to Sigma-Scientific Co. Giza, Egypt for DNA sequencing using ABI 3730xl DNA Sequence.

Computer programs used for sequence analysis

The obtained sequences files (both forward and reverse) were analyzed using a set of programs each with certain function. Staden package [19] (http://staden.sourceforge.net/) was used for nucleotide sequence assembly and formation of long contigs; FramePlot [20] (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl) was used to detect open reading frames on final contigs; Clustal W [21] (http://www2.ebi.ac.uk/clustalw) was used for amino acid alignment. Finally DNA search in the GenBank database was carried out using BLAST (Basal Local Alignment Research tool; http://www.ncbi.nlm.nih.gov/BLAST).

Results

Prevalence of the Gram negative bacterial species recovered from DFI specimens

A total of 135 Gram negative isolates representing 65.5% were recovered from 91 DFI specimens. *Proteus* spp. especially *Proteus mirabilis* were among the most common recovered Gram negative isolates (n=44/135; 32.6%) followed by

Table 1. Overall antibiotic resistance pattern of Gram negative bacterial isolates by disc diffusion method.

Antimicrobial class	Tested antimicrobial agents	No. of resistant isolates	Percentage (%)
B–lactam antibiotic	Ampicillin	135	100
	Ceftriaxone	56	41.4
	Cefotaxime	71	52.6
	Cefepime	75	55.5
	Aztreonam	74	54.8
	Imipenem	14	10.3
Quinolones	Ciprofloxacin	81	60
Aminoglycosides	Gentamicin	75	55.5
Tetracycline	Tetracycline	106	78.5

Total number of tested isolates was 135.

E. coli (n=24/135; 17.8%), followed by *Pseudomonas* spp. (n= 19/135; 14%) followed by *Klebsiella* spp. (n= 17/135; 12.6%) while other spp. such as *Citrobacter* spp., *Morganella* spp., *Providencia* spp. and *Serratia* spp. have played a minor role in bacteriology of DFIs.

Detection of ESBLs among the recovered Gram negative isolates

The overall antibiotic resistance pattern of the tested 135 isolates against various classes of antimicrobial agents by disc diffusion is summarized in **Table 1.** The results revealed that out of the 9 tested agents, the resistance pattern was equal to or exceeds 50% for 7 antimicrobial agents and only 10.3% of the tested isolates were resistant to imipenem indicating probability of ESBLs recovery from our isolates.

Potential ESBLs producing isolates as determined by disc diffusion and MIC by broth microdilution methods is summarized in **Table 2.** The results revealed that a total of 114 out of 135 were considered potential ESBL producers by disc diffusion (diameter of zone of inhibition was less than or equal to 25mm for ceftriaxone and/or diameter of zone of inhibition of cefotaxime and aztreonam was less than or equal to 27mm).

The results also revealed that 106 out of 135 were considered potential ESBL producer by showing MIC value greater than or equals to 2µg/ml for ceftriaxone. Subsequently, all the potential ESBL producers (114) were subjected to DDST and the results revealed that 58 isolates showed clavulanic acid synergy with at least one of the tested substrates (aztreonam, cefotaxime, ceftriaxone). The highest ESBL production was detected in *Proteus mirabilis*. (n= 26/33; 78.7%), followed by *Klebsiella* spp. (n=11/15; 73.3%) and finally *E. coli* (n=13/20; 65%).

Table 2. Potential ESBLs producing isolates as determined by disc diffusion[(a)] and MIC by broth microdilution[(b)] methods.

Number and % of potential ESBLs producing isolates as determined by					
Disc diffusion(a)			MIC broth microdilution(b)		
Isolate species	Number	%	Isolate species	Number	%
E. coli	20	83.3	E. coli	17	70.8
Klebsiella spp.	15	88.2	Klebsiella spp.	12	70.5
Proteus spp.	38	77.5	Proteus spp.	36	73.4
Pseudomonas spp.	19	100	Pseudomonas spp.	19	100
Others*	22	84.6	Others*	22	84.6

(a) Against ceftriaxone, cefotaxime and aztreonam. Total no. of potential producing isolates by disk diffusion was 114; (b) Against ceftriaxone. Total no. of potential producing isolates by broth dilution was 106. *others: *Citrobacter* spp., *Enterobacter* spp., *Providencia* spp. *Serratia* spp. and *Morganella* spp. % was calculated from total number of tested isolate species.

Plasmid extraction and transformation experiments

All phenotypically confirmed ESBLs producer by DDST (58 isolates) were screened for plasmids acquisition and only 8 isolates showed plasmid bands. Subsequently, transformation assay were conducted on the 8 isolates and the results revealed that plasmids extracted from *E. coli*, *Proteus mirabilis* and *Klebsiella* spp. were successfully transformed into *E. coli* DH5α, while in case of *E. coli* JM109 only 2 plasmids (extracted from two *Proteus mirabilis* isolates 26-1 and 29-1) could not be transformed.

Molecular characterization of plasmid-mediated ESBLs genes

The plasmids extracted from 3 *E. coli* isolates (28-1, 78-1 and 79-1), 4 *Proteus mirabils* isolates (26-1, 74-1, 9-1 and 72-1) and 1 *Klebsiella* pneumonia isolate (74-2) were used as templates for amplification of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes. The results revealed the apparent existence of bla_{TEM}, bla_{SHV} and bla_{CTXM} in extracted plasmids of 4 (26-1, 72-1, 78-1, 79-1); 3 (74-2, 9-1 and 79-1) and 8 (28-1, 78-1, 79-1, 26-1, 74-1, 9-1, 72-1, 74-2) of the tested isolates, respectively. For further verification of the type of ESBLs detected by PCR, DNA sequence analysis of the PCR products obtained upon using the plasmid pECDF16 extracted from the *E. coli* isolate (79-1) as a template was done. Sequence analysis of the respective PCR products revealed the presence of the three ESBL genes (bla_{TEM}, bla_{SHV} and bla_{CTX-M}) on this plasmid. The final nucleotide sequences of bla_{TEM}, bla_{SHV} located on pECDF16 plasmid were submitted to the nucleotide GenBank database under accession codes, JX976326 and JX976327 (http://www.ncbi.nlm.nih.gov/nuccore/JX976326) and JX976327 (http://www.ncbi.nlm.nih.gov/nuccore/JX976327), respectively. The results of multiple sequence alignments of bla_{TEM}, bla_{SHV} and bla_{CTX-M} with their homologous proteins using ClustalW software are depicted in **Figures 1**, **2** and **3**.

```
TEM-Ng      MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
Bla-EC      MSIQHFRVALIPFFSAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
ARP-EC      MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
TEM-128Ab    MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
TEM-1EC     MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
TEM-84EC     MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
TEM-104Kp    MSIQHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
TEM-query    MSISHFRVALIPFFAAFCLPVFAHPETLVKVKDAEDQLGARVGYIELDLNSGKILESFRP 60
         ***.**********:**********************************************

TEM-Ng      EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
Bla-EC      EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
ARP-EC      EERFPMMSTFKVLLCGAVLSRIDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
TEM-128Ab    EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
TEM-1EC     EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
TEM-84EC     EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
TEM-104Kp    EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
TEM-query    EERFPMMSTFKVLLCGAVLSRVDAGQEQLGRRIHYSQNDLVEYSPVTEKHLTDGMTVREL 120
         **********************:***************************************

TEM-Ng      CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
Bla-EC      CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
ARP-EC      CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
TEM-128Ab    CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
TEM-1EC     CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
TEM-84EC     CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
TEM-104Kp    CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
TEM-query    CSAAITMSDNTAANLLLTTIGGPKELTAFLHNMGDHVTRLDRWEPELNEAIPNDERDTTM 180
         ************************************************************

TEM-Ng      PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
Bla-EC      PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
ARP-EC      PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
TEM-128Ab    PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
TEM-1EC     PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
TEM-84EC     PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
TEM-104Kp    PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
TEM-query    PAAMATTLRKLLTGELLTLASRQQLIDWMEADKVAGPLLRSALPAGWFIADKSGAGERGS 240
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TEM-Ng      RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW 286
Bla-EC      RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW 286
ARP-EC      RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGASLIKHW 286
TEM-128Ab    RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIAEIGTSLIKHW 286
TEM-1EC     RGIIAALGPDGKPSRIVVIYTTGSQATMDERIRQIAEIGASLIKHW 286
TEM-84EC     RGIIAALGPDGKPSRIVVIYTTGSQATMDERDRQIAEIGASLIKHW 286
TEM-104Kp    RGIIAALGPDGKPSRIVVIYTTGSQATMDERNRQIVEIGASLIKHW 286
TEM-query    RGIIAALGPDGKPSRIVVIYTTGSQATMDERMSQIVDMGGSLIKHW 286
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Figure 1. Multiple alignment of amino acid sequence of TEM-query Beta-lactamase of *Escherichia coli* isolate/pECDF16 (AC=JX976326) against its homologous in the Gen Bank database: TEM-Ng = beta-lactamase, *Neisseria gonorrhoeae*, AC= NP_052173; Bla-EC= beta-lactamase, *Escherichia coli O83:H1 str.* NRG 857C; AC= YP_006162231; ARP-EC = ampicillin resistance protein, *Escherichia coli*, AC= YP_006952162; TEM-128Ab = beta-lactamase TEM-128, *Acinetobacter baumannii*, AC=AAQ57123; TEM-1EC =TEM-1 beta lactamases *Escherichia coli,* AC=ACJ43258; TEM-84EC= beta-lactamase TEM-84, *Escherichia coli*, AC=AAL29436; and TEM-104Kp =1 beta-lactamase TEM-104, *Klebsiella pneumonia*, AC= ACJ43258. The numbers indicate positions within the corresponding proteins. AC = Gen Bank accession number.

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SHV-Yp      MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE 60
SHV-95Cf     MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE 60
SHV-48kp     MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE 60
SHV-EC      MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE 60
SHV-148KL    MRYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE 60
SHV-8 query  MAYIRLCIISLLATLPLAVHASPQPLEQIKLSESQLSGRVGMIEMDLASGRTLTAWRADE 60
         * ************************************************************

SHV-Yp      RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCA 120
SHV-95Cf     RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCA 120
SHV-48kp     RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTIGELCA 120
SHV-EC      RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCA 120
SHV-148KL    RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSPVSEKHLADGMTVGELCA 120
SHV-8query    RFPMMSTFKVVLCGAVLARVDAGDEQLERKIHYRQQDLVDYSSVSEKHLAGGMTVGELCA 120
         ****************************************.*******.***:*****

SHV-Yp      AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPA 180
SHV-95Cf     AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPA 180
SHV-48kp     AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPA 180
SHV-EC      AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETERNEALPGDARDTTTPA 180
SHV-148KL    AAITMSDNSAANLLLATVGGPAGLTAFLRQIGDNVTRLDRWETELNEALPGDARDTTTPA 180
SHV-8query    AAMTMSDNRAANLLLATVGGPAGLIAFLSQIGDNVTRLDRWETEVNEALPGDARDTTTPA 180
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SHV-Yp      SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG 240
SHV-95Cf     SMAATLRKLLTSQRLSARLQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG 240
SHV-48kp     SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG 240
SHV-EC      SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG 240
SHV-148KL    SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG 240
SHV-8 query  SMAATLRKLLTSQRLSARSQRQLLQWMVDDRVAGPLIRSVLPAGWFIADKTGAGERGARG 240
         ****************** ****************************************

SHV-Yp      IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQR 286
SHV-95Cf     IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQR 286
SHV-48kp     IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQR 286
SHV-EC      IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQR 286
SHV-148KL    IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQQ 286
SHV-8 query  IVALLGPNNKAERIVVIYLRDTPASMAERNQQIAGIGAALIEHWQT 286
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Figure 2. Multiple alignment of amino acid sequence of SHV-8 query beta-lactamase of *Escherichia coli* isolate/pECDF16 (AC=JX976327) against its homologous in the GenBank database: SHV-Yp = SHV of *Yersinia pestis* biovar orientalis str. IP275, AC = YP_001102238; SHV-95Cf = SHV-95 of *Citrobacter freundii*, AC = ABN49113; SHV-48kp = SHV-48 of *Klebsiella pneumoniae*, AC = AAP03063; SHV-EC = SHV of extended-spectrum beta-lactamase of *Escherichia coli*, AC = AAO66446; SHV-148KL=SHV-148 beta-lactamase of *Klebsiella pneumoniae*, AC = AFQ23954. The numbers indicate positions within the corresponding proteins. AC = GenBank accession number.

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EC-CTX-M15    ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 120
SS-CTX-M3    ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 106
KP-CTX-M3    ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 104
SB-CTX-M3    ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 97
CTX-M3 query ---ILYRADERFAVCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 53
EnC-CTX-M    ILYRADERFAMCSTSKVMAAAAVLKKSESEPNLLNQRVEIKKSDLVNYNPIAEKHVNGTM 91
         **********:**************************************************

EC-CTX-M15    SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP 180
SS-CTX-M3    SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP 166
KP-CTX-M3    SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP 164
SB-CTX-M3    SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP 157
CTX-M-3 query SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP 113
EnC-CTX-M    SLAELSAAALQYSDNVAMNKLIAHVGGPASVTAFARQLGDETFRLDRTEPTLNTAIPGDP 151
         ************************************************************

EC-CTX-M15    RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQA-------------- 226
SS-CTX-M3    RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQA-------------- 212
KP-CTX-M3    RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPA---------- 214
SB-CTX-M3    RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPAS--------- 208
CTX-M-3 query RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTT--------------------- 152
EnC-CTX-M    RDTTSPRAMAQTLRNLTLGKALGDSQRAQLVTWMKGNTTGAASIQAGLPASWVVGDKTGS 211
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Figure 3. Partial multiple alignment of amino acid sequence of CTX-3 query beta-lactamase of *Escherichia coli* isolate/pECDF16 against its homologous in the GenBank database: EC-CTX-M15 = beta-lactamase CTX-M-15, *Escherichia coli*, AC = ACU44517; SS-CTX-M3 = extended spectrum beta-lactamase CTX-M3, *Shigella sonnei*, AC = ADY02591.1; KP-CTX-M3 = CTX-M-3 beta-lactamase, *Klebsiella pneumoniae*, AC = AAY87935; SB-CTX-M3 = extended-spectrum beta-lactamase CTX-M-3, *Shigella boydii*, AC = ABK60211; EnC-CTX-M = beta-lactamase, *Enterobacter cloacae*, AC = ABF29659.1. The numbers indicate positions within the corresponding proteins. AC = GenBank accession number.

Discussion

The present report is an extensive study regarding the phenotypic detection and molecular characterization of the most common plasmid mediated ESBLs genes recovered from Gram negative isolates causing DFIs in Egypt.

It is worth noting that the prevalence of members of Enterobacteriaceae as well as *Pseudomonas* spp. among the recovered Gram negative isolates (65.5%), have raised our fear that these pyogenic bacterial infections could be ESBLs producers. Moreover, there are multiple factors that enhance recovery of these resistant isolates, among them are overuse and misuse of antibiotic, prolonged hospital stay, being diabetic with decrease immune response and peripheral vascular diseases that limits penetration of antibiotics and hence allowing selective survival of resistant pathogens [22, 23].

With regards to CLSI, initial screening for ESBLs was performed by disc diffusion and broth microdilution. As shown in **Table 2**, a total of 114 out of 135 representing 84.4% were potential ESBL producer by disc diffusion while, a total of 106 out of 135 representing 78.5% showed MIC ≥2µg/ml for ceftriaxone. This discrepancy in results was attributed to the usage of more than one substrate such as cefotaxime, ceftriaxone and aztreonam in the first method, while in the latter case we have tested only ceftriaxone. In addition to initial screening tests, we further confirmed ESBL producers by DDST. The results revealed that 58 out of 114 tested isolates (50.8%) showed synergy with at least one of the tested substrates. Moreover, the results of the current study have drawn our attention, that besides *E. coli* [7, 22, 24] and *Klebsiella* spp.[7, 24], *Proteus* spp. especially *Proteus mirabilis*are important ESBLs producer in DFIs.

In the present study we planned to detect the prevalence of plasmid mediated ESBL producing bacteria as there is scarcity of data in our country. Almost 14% of tested isolates showed plasmid bands upon running on 0.8% agarose gel. This finding was compared with Motta et al., who detected a relatively lower percentage (6%) among Brazilians [25]. Moreover, to further confirm if these antimicrobial genes are located on plasmid or not, transformation experiments were performed. The eight tested plasmids were efficaciously transformed in *E. coli* DH5α, however only two plasmids of *Proteus mirabilis* were unsuccessfully transformed in *E. coli* JM109 and this could be attributed to their large size and/or difference in genotype of *E. coli* JM109 from that of *E. coli* DH5α which is relaxed mutant (*relA1*) characterized by production of high copy number of plasmids and hence more easily transformed.

In addition to the phenotypic screening tests for ESBLs detection, genotypic tests were done to confirm ESBL genes. PCR was used to amplify the 8 plasmid mediated ESBLs genes (bla_{SHV}, bla_{TEM}, bla_{CTX-M}). The results revealed that bla_{CTX-M} was the most common gene (100%), followed by bla_{TEM} (50%) and finally bla_{SHV} (37.5%). By surveying the literature, we recorded the increased prevalence of bla_{CTX-M} among ESBLs producer in Greece (87%) [26], Egypt (89.2%) [27] and Norway (90%) [28]. The wide spread appearance of this type of ESBLs genes could be attributed to either diversity of molecular platforms associated with it as not only plasmids but, also location of bla_{CTX-M} near or within transposons [29]. Moreover, the selective pressure exerted mainly by ceftriaxone and/or cefotaxime could be a reason behind wide spread of this type of genes [30, 31].

For further molecular characterization of variants within ESBL type, the PCR products obtained using an extracted plasmid (pECDF16) of one *E. coli* isolate (S79-1) as a template were sent for sequencing and the results revealed presence of bla_{CTX-M}, bla_{TEM-1} and bla_{SHV-8} as deposited in the GenBank. It is worth noting that many studies had reported on abundance of bla_{TEM-1} among various members of Enterobacteriaceae. It is more likely that bla_{TEM-1} will not express ESBL phenotype unless amino acids substitutions takes place at certain positions such as 104, 164, 238 and 240 [32, 33]. However, the presence of other ESBL genes such as bla_{SHV-8} and bla_{CTX-M} on the same plasmid could be the main reason for expression of ESBL phenotype. Moreover, our results revealed that this plasmid also codes for quinolones resistance, this was confirmed by successful recovery of transformed plasmid on Luria Bertani media with ciprofloxacin at final concentration 50µg/ml. Therefore, results obtained from this study are of a great clinical and environmental importance about horizontal transfer of ESBLs genes among population of clinically relevant Gram negative pathogens. Therefore, new guidelines should be undertaken in Egypt to regulate use of antimicrobial agents and hence control transfer of microbial resistance among clinically relevant pathogens.

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