academicJournals

Vol. 8(31), pp. 2947-2954, 30 July, 2014
DOI: 10.5897/AJMR2014.6909
Article Number: 575A6B946425
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Antibacterial resistance pattern of aerobic bacteria isolated from patients with diabetic foot ulcers in Egypt

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

[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., P.O. Box: 11566, Abbassia, Cairo, Egypt.

Received 19 May, 2014; Accepted 14 July, 2014

The purpose of this study was to determine and evaluate the current status of antimicrobial resistance, both phenotypically and genotypically, of the most common aerobic pathogenic bacteria recovered from diabetic foot ulcers (DFUs) in Egypt. Standard methods were used for culture identification, sensitivity testing and extended spectrum β-lactamases (ESBLs) detection. PCR for *bla* genes was performed and the obtained PCR products were verified by DNA sequencing. A total of 206 clinical bacterial isolates were recovered from DFU specimens, of which 135 (65.5%) were Gram negative and 71 (34.5%) were Gram positive. Gram negative isolates were mainly *Proteus* spp. (49; 24.4%), *Escherichia coli* (24; 11.6%), *Pseudomonas* spp. (19; 9.2%) and *Klebsiella* spp. (17; 8.2%) while, Gram positive isolates were mostly *Staphylococcus aureus* (26; 12.6%) and coagulase-negative staphylococci (25; 12.13%). The antibiogram analysis of Gram negative isolates revealed a remarkable high resistance pattern towards most of the tested antibiotics particularly, 3rd, 4th generation cephalosporins and fluroquinolones. About, 50.87% of Gram negative isolates were ESBL producers of which 14% were plasmid-mediated. Upon molecular characterization of plasmid-mediated ESBLs by PCR, bla_{CTX-M} showed 100% positivity, followed by bla_{TEM} (50%) and bla_{SHV} (37.5%). For further detection of variants within genes, only one *E. coli* isolate that harbored a plasmid coding for three genes was sent for DNA sequencing. The results revealed presence of bla_{TEM-1} (accession number JX976326), bla_{SHV-8} (accession number JX976327) and bla_{CTX-M} on a single plasmid coded pECDF16 as deposited in GenBank. The majority of ESBLs recovered from DFUs, showed resistance to more than one class of antimicrobial agents and hence new guidelines should be addressed in Egypt to rationalize and prevent the misuse and overuse of antimicrobial agents.

Key words: Diabetic foot ulcers, extended spectrum β-lactamase (ESBL), plasmid, bla_{CTX-M}, bla_{TEM-1}, bla_{SHV-8}.

INTRODUCTION

The estimated prevalence of diabetes mellitus in Egypt had significantly increased from 7.3 million in 2011 to 12.4 million in 2030 (Whiting et al., 2011). The burden of the disease is attributed mainly to development of diabetes chronic complications. Diabetic foot infections (DFIs) are one of the most serious medical complications that require proper diagnosis and therapeutic strategies (Hobizal and Wukick, 2012). Usually 15% of diabetic patients develop an ulcer that is highly susceptible to an infection. DFIs bacteriological studies are unique as

infection can be caused by Gram positive and/or Gram negative aerobic and anaerobic bacteria as monomicrobial or polymicrobial bacteria (Edmonds, 2006). Moreover, the spectrum of bacterial species that had been recovered from limb threatening DFIs, can change dramatically in case of hospitalized patient, surgical procedures and prolonged broad spectrum antibiotic uptake. The frequency of extended spectrum β-lactamase (ESBLs) producing Enterobacteriaceae is increasing worldwide among DFIs isolates (Zubair et al., 2011).

ESBLs are a heterogeneous group of plasmid-mediated β-lactamases. This group confers resistance to oxyimino-cephalosporins especially 3rd generation as well as aztreonam, an example of oxyimino- monobactams (Bradford, 2001). ESBLs are generally inhibited by β-lactamase inhibitor. Plasmid-mediated ESBLs usually show co-resistance to other classes of antibiotics such as quinolones and aminoglycosides, hence limiting therapeutic options (Jacoby and Sutton, 1991, Varaiya et al., 2008). ESBLs of clinically encountered importance belong mainly to TEM-, or SHV-derivatives and/or CTX-M like enzymes. So far (uptill June 2013), up to three hundred and eighty eight variants of TEM/SHV type enzyme were reported by Lahey website (http://www.lahey.org/Studies/) and up to five groups of CTX-M have been characterized based on amino acid sequences (Bonnet, 2004; Poirel et al., 2012). The gigantic genetic diversity among the widely spread enzymes made it absolutely necessary to perform extensive genotypic studies, in addition to detailed phenotypic screening tests for better correlation of results.

Numerous studies all over the world had reported on the most common bacterial isolates involved in DFIs along with their *in vitro* susceptibility pattern. However, most of these reviews showed a wide range of microbial etiology as well as sensitivity patterns (Hatipoglu et al., 2014). The proper management of these infections depend on appropriate selection of antibiotics based on antibiograms. Moreover, prescribing initial empirical therapy is usually correlated to previous knowledge about sensitivity pattern in this region. Accordingly, the goal of this study is to detect the prevalence of antimicrobial resistance among the most common aerobic bacterial pathogens associated with DFUs in Egypt.

MATERIALS AND METHODS

Specimen processing

A total of one hundred and two DFU specimens were collected during the period of November 2010 to February 2012 from different hospitals and clinics located in Cairo, Egypt. After washing the ulcer with sterile physiological saline, sterile cotton swabs were introduced to the base of the ulcer and were subsequently inserted in Amies transport media to maintain specimen during transportation to the laboratory. Firstly, swabs were used for specimen isolation on primary media such as nutrient agar, blood agar and MacConkey agar and secondly, for Gram staining. Isolates were identified to the genus and/or species level by macroscopical, microscopical and conventional standard biochemical tests according to Bergey's manual of determinative bacteriology, 1994 (Bergey and Holt, 1994).

Antimicrobial sensitivity test and phenotypic detection of ESBLs

After identification of tested isolates, antimicrobial susceptibility testing of aerobic and facultative anaerobes isolates was performed by Kirby Bauer disc diffusion method as recommended by Clinical and Laboratory Standard Institute (CLSI, 2010-2011). The choice of the antimicrobial agents depended mainly on the commonly encountered empiric antibiotic regimen in Egypt. The tested agents for Gram negative isolates were ampicillin, aztreonam, cefotaxime, ceftriaxone, cefepime, ciprofloxacin, gentamicin and imipinem (Oxoid, England). In addition to the previous tested agents, vancomycin and clindamycin were tested on Gram positive isolates.

Based on CLSI, 2010 potential ESBLs were considered if ceftriaxone reading was ≤ 25mm, while cefotaxime or aztreonam reading was ≤ 27mm. For further phenotypic determination of ESBLs modified double disc synergy test (DDST) was performed as described by Jarlier et al. (1988). A disc of amoxicillin/clavulanic acid was placed in the middle surrounded by disc of ceftriaxone, cefotaxime, cefepime and aztreonam (discs were placed at distance of 20 mm center to center from amoxicillin/clavulanic acid). Positive results were indicated by the enhancement of inhibition zone of cephalosporin discs towards the clavulanic acid disc. Minimum inhibitory concentration (MIC) of ceftriaxone was measured by microbroth dilution methods according to the recommendations of CLSI. ESBL producing *Escherichia coli*, *Klebsiella pneumonia* and *Proteus mirabilis* were indicated by MIC reading ≥ 2 µg/ml for ceftriaxone.

Plasmid extraction

Preparation of plasmid DNA from *E. coli* was performed according to alkaline-SDS method (Birnboim and Doly, 1979). An overnight culture of the tested isolates was grown on Luria Bertani (LB) broth containing ampicillin 100 µg/ml. Plasmids were detected and separated using agarose gel electrophoresis which was carried out essentially as described by Sambrook and Russell (2001).

Transformation experiments

E. coli DH5α (Hanahan, 1983) or *E. coli* JM109 (DE3) (Novagen, Darmstadt) competent cells were prepared according to the modified Hanahan (1983) method and were transformed according to Sambrook and Russell (2001). In general 10 µl of the obtained plasmid containing 0.01 - 1.0 µg DNA were added to 200 µl

*Corresponding author. E-mail: aboshanab2003@yahoo.com or aboshanab2012@pharma.asu.edu.eg.

Table 1. Prevalence of the aerobic/facultative anaerobic bacterial species recovered from diabetic foot ulcer specimens.

Gram negative bacteria	Total no.	%	Gram positive bacteria	Total no.	%
Proteus mirabilis	44	21.3	*Staphylococcus aureus*	26	12.6
Proteus vulgaris	4	1.9	CONS (*Staphylococcus epidermidis)*	25	12.13
Proteus penneri	1	0.5	*Streptococcus* spp.	5	2.4
E. coli	24	11.6	*Corynebacterium* spp.	9	4.3
*Klebsiella s*pp.	17	8.2	*Micrococcus* spp.	6	2.9
Pseudomonas spp.	19	9.2	Total number	71	34.46
Citrobacter spp.	7	3.3			
Enterobacter spp.	4	1.9			
Morganella spp.	7	3.3			
Providencia spp.	4	1.9			
Serratia spp.	4	1.9			
Total number	135	65.53			

competent cells and kept on ice for 30 min. Following a heat shock (90 sec, 42°C), cells were regenerated in 800 µl SOC or LB medium for 1 h at 37°C, 300 rpm then the cell suspension was plated out on LB agar plates containing the appropriate antibiotic.

DNA amplification by PCR

The extracted plasmids of the phenotypically confirmed potential ESBLs producer were used as templates for PCR. The primers used for detection of: (i) bla_{TEM} (Rasheed et al., 1997) (0.9 kb) were BlaTEM-F: 5'- ATGAGTATTCAACATTTCCG-3' and BlaTEM-R: 5'-CTGACAGTTACCAATGCTTA-3'; (ii) for bla_{SHV} (Rasheed et al., 1997) (0.8 kb) were BlaSHV-F: 5'-GGTTATGCGTTATATTCGCC-3' and BlaSHV-R: 5'- TTAGCGTTGCCA GTGCTC-3'; and (iii) for bla_{CTX} (Bonnet *e*t al., 2001) (0.5 kb) were BlaCTX-m-F: 5'-CGC TTT GCG ATG TGC AG-3, BlaCTX-m-R: 5'-ACC GCG ATA TCG TTG GT-3'. PCR was performed in a Nyx-Technik Inc. Personal Cycler (ATC401, USA). Each assay (50 µl) consisted of 200 ng DNA, 25 pmole of each appropriate primer, 0.2 mM dNTPs (Invitrogen, Karlsruhe, Germany), 3 mM $MgCl_2$, and 2 U *Taq* DNA polymerase (Sigma, USA). The cycling parameters compromised initial denaturation of the target DNA sequence at 95°C for 2 min (1 cycle), denaturation at 95°C for 30 s (30 cycles), annealing of both forward and reverse primers at 47°C for 45 s (30 cycles), extension at 72°C for 90 s (30 cycles) and a final extension step at 72°C for 5 min (1 cycle).

Sequencing of the PCR products

The PCR products were purified using QIA quick Gel Extraction Kit (Qiagen, Hilden) according to the manufacture's specifications and were sent to Sigma-Scientific Co. Giza, Egypt for DNA sequencing using ABI 3730xl DNA Sequencer. The obtained nucleotide sequences files were further assembled and analyzed using the Staden Package program version 4 (Staden, 1996) (http://staden.sourceforge.net/). The final assembled sequence was submitted to GenBank and the accession numbers of the respective submitted sequences were obtained.

Nucleotide accession codes

The nucleotide sequences reported in this study was deposited in GenBank databases under the accession codes JX976326 and JX976327.

RESULTS

Bacteriology of DFIs

Out of the 102 specimens, 91 (89.2%) showed positive culture whereas, 11 (10.7%) showed negative culture. From positive culture specimens, 12 (13.18%), 43 (47.25%) and 36 (39.56%), one, two or three bacterial isolates were recovered from each one, respectively. A total of 206 clinical bacterial isolates were recovered, of which 135 (65.5%) were Gram negative and 71 (34.5%) were Gram positive.

As shown in Table 1, the predominant recovered bacteria were members of Enterobacteriaceae (116 out of 206, 56.3%). *Proteus* spp. was among the most common recovered Gram negative isolates as they represented 24.4% followed by *E. coli*, *Pseudomonas* spp. and *Klebsiella* spp. which accounted for 11.6, 9.2 and 8.2%, respectively. Both *Citrobacter* spp. and *Morganella* spp. accounted for 6.6% of the total collected isolates. Each of *Enterobacter* spp., *Serratia* spp. and *Providencia* spp. represented 2% of the total isolates. On the other hand, the most common Gram positive bacteria were *Staphylococcus aureus* and coagulase negative staphylococci (CONS) (12.1-12.6%). *Corynebacterium* spp*., Micrococcus* spp. and *Streptococcus* spp. were involved with 2.4- 4.3% isolation rate.

Antibiogram analysis of Gram positive and negative isolates

The antimicrobial resistance pattern of Gram positive and negative isolates are summarized in Tables 2 and 3. All

Table 2. Antimicrobial resistance pattern of recovered Gram positive cocci isolates.

Antimicrobial agent	*Staphylococcus aureus*		CONS and *Micrococcus* spp.		*Streptococcus* spp.	
	No=26	(%)	No=25	(%)	No=5	(%)
Ampicillin	26	(100)	31	(100)	0	0
Amoxicillin-clavulanic acid	11	(42.3)	4	(12.9)	Not tested	
Cephalexin	20	(76.9)	12	(38.7)	0	0
Oxacillin	8	(30.7)	1	(3.2)	Not tested	
Vancomycin	1	(3.8)	0	0	0	0
Cefuroxime	11	(42.3)	10	(32.2)	0	0
Gentamicin	8	(30.7)	7	(22.5)	Not tested	
Tetracycline	22	(84.6)	16	(51.6)	2	(40)
Clindamycin	6	(23)	3	(9.6)	0	0

(%) Percentage of resistant isolates; CONS: coagulase negative Staphylococci.

Table 3. Antimicrobial resistance pattern of recovered Gram negative rods.

Antimicrobial agent	*Proteus* spp.		*E. coli*		*Pseudomonas* spp.		*Klebsiella* spp.		Others*	
	No=49	(%)	No=24	(%)	No=19	(%)	No=17	(%)	No=26	(%)
Ampicillin	49	(100)	24	(100)	19	(100)	17	(100)	26	(100)
Ceftriaxone	21	(42.8)	11	(45.8)	10	(52.6)	10	(58.82)	4	(15.3)
Cefotaxime	27	(55.1)	13	(54.8)	13	(68.4)	11	(64.7)	7	(26.9)
Cefepime	29	(59.1)	11	(45.8)	16	(84.2)	8	(47)	11	(42.3)
Ciprofloxacin	25	(51)	19	(79.1)	12	(63.1)	12	(70.5)	13	(50)
Aztreonam	30	(61.2)	14	(58.3)	11	(57.8)	9	(52.9)	10	(38.4)
Imipenem	8	(16.3)	1	(4.16)	1	(5.2)	3	(17.6)	1	(3.84)
Gentamicin	28	(57.1)	8	(33.3)	16	(84.2)	7	(41.17)	16	(61.5)
Tetracycline	46	(93.8)	13	(54.1)	19	(100)	11	(64.7)	17	(65.3)
Piperacillin-tazobactam	Not tested		Not tested		15	(78.9)	Not tested		Not tested	

(%) percentage of resistant isolates; Others* include *Citrobacter* spp., *Enterobacter* spp., *Morganella* spp., *Providencia* spp. and *Serratia* spp.

S. aureus and coagulase negative staphylococci isolates were 100% resistant to ampicillin by disc diffusion method. However, vancomycin showed high sensitivity pattern towards both *S. aureus* (96.1%) and coagulase negative *Staphylococci* isolates (100%). The majority of streptococci isolates were sensitive to ampicillin and cefuroxime (100% each), cephalexin and clindamycin (80% each) (Table 2).

The overall antibiotic resistance pattern of Gram negative isolates revealed that out of 135 isolates, 55.5, 54.8 52.6 and 41.4% were resistant to cefepime, aztreonam, cefotaxime and ceftriaxone, respectively. Moreover, the antibiogram results revealed that tested isolates showed co-resistance with other classes of antimicrobial agents such as tetracycline (78.5%), ciprofloxacin (60%) and gentamicin (55.5%). Imipinem was active against more than 89.7% of the tested isolates (Table 3). Based on the previously mentioned antibiogram results, it becomes important to search for the detection and assessment of ESBLs among Gram negative isolates.

Phenotypic detection of ESBLs

Based on CLSI guidelines 2010-2011, disc diffusion and microbroth dilution methods were used to screen for potential ESBL producers. The results revealed that a total of 114 out of 135 (84.4%) isolates were considered as potential ESBL producers by disc diffusion method

Table 4. Summarization of phenotypic and genotypic character of the plasmid-mediated ESBL producing isolate.

Isolate species and code	Antibiotic resistance pattern of wild type	Molecular detection of ESBLs (PCR)	Transformation experiment into		Antibiotic resistance pattern exhibited by transformants
			E. coli DH5α	*E. coli* JM109(DE3)	
E. coli (S28-1)	AMP, CRO, CTX, CIP, ATM, CN, TE	bla_{CTX-M}	+	+	AMP, CRO, CTX, ATM
Klebsiella spp. (S74-2)	AMP, CRO, CTX, FEP, CIP, ATM, CN, TE	bla_{SHV},bla_{CTX-M}	+	+	AMP, CRO, CTX, ATM
Proteus mirabilis (S26-1)	AMP, CRO, CTX, FEP, CIP, ATM, CN, TE, IMP	bla_{TEM},bla_{CTX-M}	+	-	AMP, CRO, CTX, ATM
Proteus mirabilis (S74-1)	AMP, CRO, CTX, FEP, CIP, ATM, CN, TE	bla_{CTX-M}	+	+	AMP, CRO, CTX, ATM, FEP
Proteus mirabilis (S9-1)	AMP, CRO, CTX, FEP, CIP, ATM, CN, TE, IMP	bla_{SHV}, bla_{CTX-M}	+	-	AMP, CRO, CTX, ATM,
Proteus mirabilis (S72-1)	AMP, CTX, FEP, CIP, ATM, CN, TE	bla_{TEM}, bla_{CTX-M}	+	+	AMP, CRO, CTX, ATM
E. coli (S78-2)	AMP, CRO, CTX, FEP, CIP, ATM, CN, TE	bla_{TEM}, bla_{CTX-M}	+	+	AMP, CRO, CTX, ATM
E. coli (S79-1)	AMP, CRO, CTX, CIP, ATM, CN, TE	bla_{SHV}, bla_{TEM}, bla_{CTX-M}	+	+	AMP, CRO, CTX, CIP, ATM

AMP = ampicillin, CRO = ceftriaxone, CTX = Cefotaxime, FEP = cefipime, CIP = ciprofloxacin, ATM = aztreonam, IMP = imipenem, CN = gentamicin, TE = tetracycline.

while, a total of 106 out of 135 (78.51%) showed MIC value greater than or equal 2 µg/ml for ceftriaxone. By subsequently performing phenotypic DDST, a total of 58 out of 114 (50.8%) were positive. The highest ESBL production was detected in *Klebsiella* spp. (11 out of 15; 73.33%), followed by *Proteus* spp. (27 out of 38; 71%) and *E. coli* (13 out of 20; 65%) while other Gram negative isolates such as *Pseudomonas*, *Citrobacter* and *Providencia* species recorded relatively low number of ESBL producing isolates.

Plasmid extraction, detection and transformation

A total of 8 out of 58 isolates (13.8%) that were previously screened to be ESBL producers by DDST showing bands in their electrophoresed plasmid extract. Four out of 26 (15.38%) of *Proteus* spp., 3 out of 13 (23.67%) of *E. coli* and only one out of 11 (9%) *Klebsiella* spp. were found to harbor plasmids of different sizes. Results of plasmid transformation of the respective isolates into competent *E. coli* DH5α and *E. coli* JM109(DE3) host strains revealed that all the tested plasmid extracts were successfully transformed into the former competent cell while, only two plasmid extracts of *Proteus mirabilis* were not transformed with the latter competent cell (Table 4).

Molecular characterization of ESBLs gene by PCR and sequencing

All the plasmid mediated ESBLs producers were examined by PCR for the presence of bla_{SHV}, bla_{TEM} and bla_{CTX-M} using the obtained plasmid DNA as a template. PCR results revealed that bla_{CTX-M} was the most common gene (all tested eight isolates), followed by bla_{TEM} (four out of eight isolates) and finally bla_{SHV} (three out of eight isolates). Two or more ESBLs gene were present in 6 (75%) of 8 ESBL isolates, bla_{TEM} + bla_{CTX-M} was the most common combination (3/8, 37.5%), followed by bla_{SHV} + bla_{CTX-M} (2/8, 25%) and bla_{TEM} + bla_{SHV} + bla_{CTX-M} (1/8, 12.5%). Nucleotide sequence analysis of the amplified PCR products confirmed the presence of bla_{TEM-1}, bla_{CTX-M} and bla_{SHV-8} genes carried on one plasmid named pECDF16 in one of ESBL producing *E. coli* clinical isolate. After transformation of this plasmid into *E. coli* DH5α or *E. coli* JM109 (DE3) host strains, the same antibiotic resistant profile of the wild *E. coli* isolate was reserved (Table 4).

DISCUSSION

The current study was aimed to determine and evaluate the current status of antimicrobial resistance both phenotypically and genotypically for the most common aerobic pathogenic bacteria recovered from diabetic foot ulcers (DFUs).

According to the high number of Egyptian diabetic patients suffering from foot infections, the current study shows a clear understanding of correlation and correspondence in the order of results between phenotypic and genotypic studies of antimicrobial resistance of aerobic

bacteria associated with DFIs.

Diversity of bacterial aetiology of DFIs has been well documented in the literature. In the present study, polymicrobial cultures compromised about 87% of specimens with an average rate of isolation of 2.26 aerobic bacteria per positive specimen, which is slightly lower than that reported by Citron et al. (2007). Being chronic long standing ulcers, DFU are more vulnerable to polymicrobial infection with co-existence of Gram negative aerobic and/or anaerobic bacteria (Lipsky et al., 2006, Omar et al., 2008). Though previous studies reported that Gram positive aerobes were the predominant organisms among DFIs (Bamberger et al., 1987, Citron et al., 2007, Mendes et al., 2012), the current study found that Gram negative bacteria especially members of Enterobacteriaceae were the predominant one. Our results were in correspondence with other studies that were conducted mainly in developing countries, which showed that Gram negative bacteria lie between 51.4 and 80% (Gadepalli et al., 2006; Paul et al., 2009). The predominance of aerobic Gram negative bacilli in our study could be related to the severity of cases studied; this also explains the reason behind low incidence of examined Gram positive cocci. Moreover, in developing countries, diabetic patients usually consult physician in the case of severe complicated ulcers, while on the other hand patients in developed countries contact their health care team as soon as they observe an ulcer. The consequence of delayed medical advice in Egypt may be responsible for predominance of the Gram negative aerobic bacteria in polymicrobial infection.

Overall, *P. mirabilis* were the most common isolated pathogen (22%), followed by *E. coli* (11.6%), *Pseudomonas* spp. (9%) and *Klebsiella* spp. (8%) of total isolates. Similar studies, conducted by several investigators reported the predominance of *Proteus* spp. among DFIs isolates (Ramani et al., 1991, Ako-Nai AK et al., 2006).

However, a study presented by Chincholikar and Pal (2002) showed that *Pseudomonas* spp. were the most common isolates while, other study conducted by Dezfulian et al. (2011) highlighted role of *E. coli* and *Klebsiella* spp. in causing pyogenic bacterial infections. We attributed these discrepancies in results of the geographical variation, type of the ulcer and the severity of the infections which were included in the studies (Citron et al., 2007). Thus a universal diabetic wound ulcer grading system will be of immense importance in comparing microbilogical results of DFIs.

It is a matter of concern that members of Enterobacteriaceae as well as *Pseudomonas* spp. showed a notable high rate of resistance pattern towards commonly tested antibiotics especially 3rd generation cephalosporins and aztreonam by disc diffusion method, which thereby, directed us to screen of ESBLs among isolates. The overall rate of phenotypic screening by DDST and microbroth dilution revealed 50.8 and 67% as potential ESBL producers, respectively. Despite corelatively limited data on prevalence of ESBLs among DFIs, most studies have reported on growing threat of ESBLs producer among *E. coli, Klebsiella* spp. (Gadepalli et al., 2006; Varaiya et al., 2008), *Proteus* spp. and other Gram negative rods (Zubair et al., 2011). The increased prevalence of multiple drug resistant ESBLs among DFIs in developing countries is more likely to be attributed to multiple factors including overuse and misuse of antibiotics, prolonged hospital stay, being diabetic as well as the associated peripheral vascular disease that limits penetration of antibiotics to ulcer site thereby enhancing selection of resistant isolates(Hartemann-Heurtier et al., 2000, Gadepalliet al., 2006).

In the current study, the overall prevalence rate of plasmid-mediated ESBLs among DFIs isolates was almost 14%. Lower detection rate (6%) than ours was reported by Motta et al. (2003).

Therefore, we believe that strict preventive measures are required to overcome dissemination of antibiotic resistance genes via plasmids. Moreover, transformation experiments were done to further confirm whether the resistant gene were located on plasmids or not via studying whether the trans-formable host strains retain all or some of the phenotypic resistant pattern of wild isolate. Results show that eight plasmids were successfully transformed into *E. coli* DH5α host strains however, two of them were unsuccessfully transformed into *E. coli* JM109 (DE3) host strains particularly because of their larger sizes.

Notably, since the majority of plasmid mediated ESBLs are derived by point mutation (from the common TEM-1 and SHV1/SHV-2 β-lactamases) (Jacoby and Sutton 1991; Bush et al., 1995; Gniadkowski et al., 1998), hence molecular characterization of these ESBLs will be of significance importance in our country to trace variant/novel ESBL genotypes. Therefore, all phenotypically confirmed plasmid mediated ESBL (eight isolates) were subjected to PCR using bla_{TEM}, bla_{SHV} and bla_{CTX-M} specific primers. Overall, bla_{CTX-M} was found to be the most prevalent ESBL detected among eight isolates (100%), followed by bla_{TEM} gene (50%) and bla_{SHV} (37.5%). Zubair et al. (2012) reported that bla_{CTX-M} was the most prevalent ESBL among DFU in India (82%), followed by bla_{TEM} (50%) and bla_{SHV} (46.9%) (Zubair et al., 2012). These slightly observed differences may be due to limitation of their study to cefotaxime resistant *E. coli* and *Klebsiella* spp. only.

Interestingly, the three ESBL resistant genes bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ were detected in the plasmid preparation of the multidrug resistant *E. coli* isolate (S79-1). This was confirmed by DNA sequencing of the respective amplicons obtained from PCR (after clean up). By submitting the sequences to be deposited in GenBank, the plasmid preparation was given the code

pECDF16 while, the sequences of bla_{TEM-1} and bla_{SHV-8} were under accession numbers AC = JX976326 and AC = JX976327, respectively in GenBank database. Although, TEM-1 is not considered an ESBL phenotype, unless amino acid substation at certain positions take place (Gniadkowski, 2008). Interestingly, the association of $bla_{SHV-8}$with bla_{TEM-1} is more likely to be responsible for expression of ESBL phenotype. Moreover, our results revealed that the respective plasmid confers resistance not only to ampicillin, 3rd generation cephalosporin and aztreonam, but also to other antibacterial agents such as ciprofloxacin. This means that both quinolones and ESBL resistant genes were more likely to coexist on the same plasmid. Demonstration of high levels existence of mobile genes conferring reduced susceptibility to fluroquinolones as well as the presence of ESBL genes in fish produced in China and identification of a significant reservoir of antibiotic resistance genes relevant to human medicine was detected by Jiang et al. (2012). Therefore, these findings are of a great clinical and environmental importance for horizontal transfer of various resistant genes among population of clinically relevant pathogens. Therefore, new guidelines should be addressed in the developing countries to decrease or prevent the misuse and abuse of antimicrobial agents since the issue of antibiotic resistant is rapidly growing particularly in diabetic foot infections as confirmed in this study.

Conclusion

The antibiogram analysis of Gram negative isolates isolated from patients with diabetic foot ulcers showed a remarkable high resistance pattern towards most of the tested antibiotics particularly, 3rd, 4th generation cephalosporins and fluroquinolones. About, 50.87% of isolates were ESBL producers of which 14% were plasmid-mediated. Therefore, new guidelines should be addressed in Egypt to rationalize and prevent the misuse and overuse of antimicrobial agents.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ako-Nai AK, Ikem IC, Akinloye OO, Aboderin, Ikem R, Kassim O (2006). Characterization of bacterial isolates from diabetic foot infections in Ile-Ife, Southwestern Nigeria. Foot 16(3): 158-164.

Bamberger DM, Daus GP, Gerding GN (1987).Osteomyelitis in the feet of diabetic patients. Long-term results, prognostic factors, and the role of antimicrobial and surgical therapy. Am. J. Med. 83(4): 653-660.

Bergey DH, Holt JG (1994). Bergey's manual of determinative bacteriology. Baltimore, Williams & Wilkins.

Birnboim HC, Doly J (1979).A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7(6):1513-1523.

Bonnet R (2004).Growing Group of Extended-Spectrum β-Lactamases: the CTX-M Enzymes. Antimicrob. Agents Chemother. 48(1):1-14.

Bonnet RC, Dutour JL, Sampaio MC, Chanal CD, Sirot D, Labia RJ, Sirot J (2001). Novel cefotaximase (CTX-M-16) with Increased catalytic efficiency due to substitution Asp-240→Gly. Antimicrob. Agents Chemother. 45(8):2269-2275.

Bradford PA (2001). Extended-Spectrum β-Lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat. Clin. Microbiol. Rev. 14(4):933-951.

Bush K, Jacoby GA, Medeiros AA (1995).A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39(6):1211-1233.

Chincholikar D, Pal R (2002).Study of fungal and bacterial infections of the diabetic foot. Indian J. Pathol. Microbiol. 45(1):15-22.

Citron DM, Goldstein JC, Merriam CV, Lipsky BA, Abramson MA (2007). Bacteriology of Moderate-to-Severe Diabetic Foot Infections and *In Vitro* Activity of Antimicrobial Agents. J. Clin. Microbiol. 45(9):2819-2828.

Clinical and Laboratory Standards Institute (CLSI) (2010). Performance standards for antimicrobial susceptibility testing; twentieth informational supplement CLSI document M100-S20, Clinical and Laboratory Standards Institute , Wayne, PA.

Clinical and Laboratory Standards Institute (CLSI) (2011). Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement. CLSI document M100-S21, Clinical and Laboratory Standards Institute, Wayne, PA.

Dezfulian A, Salehian MT, Amini V, Dabiri H, Azimi Rad M, Aslani MM, Zali MR (2011). Bacteriological study of diabetic foot infections in an Iranian hospital. Iran Red Crescent Med. J. 13(8):590-591.

Edmonds M (2006).Diabetic foot ulcers: practical treatment recommendations. Drugs 66(7):913-929.

Gadepalli R, Dhawan B, Sreenivas V, Kapil A, Ammini AC, Chaudhry R (2006).A Clinico-microbiological Study of Diabetic Foot Ulcers in an Indian Tertiary Care Hospital. Diabetes Care 29(8):1727-1732.

Gniadkowski M (2008).Evolution of extended-spectrum beta-lactamases by mutation. Clin. Microbiol. Infect. 14 Suppl. 1:11-32.

Gniadkowski M, Schneider I, Jungwirth R, Hryniewicz, W, Bauernfeind A (1998). Ceftazidime-resistant Enterobacteriaceae isolates from three polish hospitals: Identification of three novel TEM- and SHV-5-Type extended-spectrum β-lactamases. Antimicrob. Agents Chemother. 42(3):514-520.

Hanahan D (1983).Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166(4):557-580.

Hartemann-Heurtier A, Marty L, Ha Van G, Grimaldi A (2000).Role of antibiotic therapy in diabetic foot management. Diabetes Metab. 26(3):219-224.

Hatipoglu M, Mutluoglu M, Uzun G, Karabacak E, Turhan V, LipskyBA (2014) .The microbiologic profile of diabetic foot infections in Turkey: a 20-year systematic review: diabetic foot infections in Turkey. Eur. J. Clin. Microbiol. Infect. Dis. 33(6):871-878.

Hobizal KB, Wukich DK (2012). Diabetic foot infections: current concept review. Diabet. Foot Ankle 3.

Jacoby GA, Sutton L (1991).Properties of plasmids responsible for production of extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. 35(1):164-169.

Jarlier V, Nicolas MH, Fournier G, Philippon A (1988). Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev. Infect. Dis. 10(4):867-878.

Jiang HX, Tang D, Liu YH, Zhang XH, Zeng ZL, Xu L, Hawkey PM (2012).Prevalence and characteristics of beta-lactamase and plasmid-mediated quinolone resistance genes in *Escherichia coli* isolated from farmed fish in China. J. Antimicrob. Chemother. 67(10):2350-2353.

Lipsky BA, Berendt AR, Deery HG, Embil JM, Joseph WS, Karchmer AW, Tan JS (2006).Diagnosis and treatment of diabetic foot infections. Plast. Reconstr. Surg. 117(7 Suppl.): 212S-238S.

Mendes JJ, Marques-Costa A, Vilela C, Neves J, Candeias N, Cavaco-Silva P, Melo-Cristino J (2012). Clinical and bacteriological survey of diabetic foot infections in Lisbon. Diabetes Res. Clin. Pract. 95(1):153-161.

Motta RN, Oliveira MM, Magalhaes PS, Dias AM, Aragao LP, Forti AC, Carvalho CB (2003).Plasmid-mediated extended-spectrum beta-lactamase-producing strains of Enterobacteriaceae isolated from diabetes foot infections in a Brazilian diabetic center. Braz. J. Infect. Dis. 7(2):129-134.

Omar NS, El-Nahas MR, J. Gray J (2008).Novel antibiotics for the management of diabetic foot infections. Int. J. Antimicrob. Agents 31(5):411-419.

Paul S, Barai L, Jahan A (2009).A bacteriological study of diabetic foot infection in an urban tertiary care hospital of Dhaka city. Ibrahim Med. Coll. J. 3(2):50-54.

Poirel LR, Bonnin RA, Nordmann P (2012).Genetic support and diversity of acquired extended-spectrum β-lactamases in Gram-negative rods. Infect. Genet. Evol. 12(5):883-893

Ramani A, Ramani R, Shivananda PG, Kundaje GN (1991). Bacteriology of diabetic foot ulcers. Indian J. Pathol. Microbiol. 34(2): 81-87.

Rasheed JK, Jay C, Metchock B, Berkowitz F, Weigel F, Crellin J, Tenover FC (1997).Evolution of extended-spectrum beta-lactam resistance (SHV-8) in a strain of *Escherichia coli* during multiple episodes of bacteremia. Antimicrob. Agents Chemother. 41(3):647-653.

Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press.

Staden R (1996).The Staden sequence analysis package. Mol. Biotechnol. 5(3):233-241.

Varaiya AY, Dogra JD, Kulkarni MH, Bhalekar PN (2008).Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in diabetic foot infections. Indian J. Pathol. Microbiol. 51(3):370-372.

Whiting DR, Guariguata L, Weil C, Shaw J (2011).IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res. Clin. Pract. 94(3):311-321.

Zubair M, Malik A, Ahmad J (2011).Clinico-microbiological study and antimicrobial drug resistance profile of diabetic foot infections in North India. Foot (Edinb) 21(1):6-14.

Zubair M, Malik A, Ahmad J (2012).Study of plasmid-mediated extended-spectrum beta-lactamase-producing strains of enterobacteriaceae, isolated from diabetic foot infections in a North Indian tertiary-care hospital. Diabetes Technol. Ther. 14(4):315-324.