

Full Length Research Paper

Occurrence of multiple antibiotic resistance phenotype and class 1 integron in bacteria isolated from diabetic foot ulcers

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Resistance profile to antibiotics and the occurrence of class-1 integron in 23 bacteria isolated from diabetic foot ulcers (DFUs) were analyzed. Among 23 isolates, 16 elicited resistance to at least two antibiotics and seven were sensitive. Majority of the isolates were resistant to spectinomycin and streptomycin (68.75%) followed by trimethoprim (62.50%) and ampicillin (50%). Among the antibiotics resistant isolates, six exhibited resistance to four different groups of antibiotics and one showed resistance to 11 antibiotics (six antibiotic groups). 17 bacterial strains possessed class-1 integrons with an amplicon size of 0.30 to 2.40 kb which suggested their role in conferring resistance to antibiotics. 2-D gel electrophoresis of proteins extracted from *Escherichia coli* DF39TA grown with antibiotics revealed significant alteration in total proteome as compared to control culture. Five spots showing four fold up regulation were identified by MALDI TOF MS as OmpX, OmpA, OmpA-OmpF, Omp-toIC and chaperone protein DnaK. Changes in abundance of above proteins following growth with antibiotics may be beneficial for antibiotic resistant bacteria to adapt under adverse environmental conditions. Findings of this study suggest that infection with multiple antibiotic-resistant bacteria is common to DFUs and resistance is mediated by class-1 integrons.

Key words: Diabetic foot ulcer, antibiotic resistance, polymerase chain reaction (PCR), class-I integron, 2-D gel electrophoresis, *Escherichia coli*.

INTRODUCTION

Diabetic foot infection is a common and potentially devastating complication that can progress rapidly to irreversible septic gangrene necessitating amputation of the foot. All diabetic foot ulcers (DFUs) are contaminated with a variety of organisms which are aerobic Gram-negative bacilli, anaerobes, and certain Gram-positive

species (Gadepalli et al., 2006; Singh et al. 2009). The specific organisms found in diabetic foot infections differ not only from patient to patient, but also from one part of the country to another (Ozer et al., 2010). Individuals with diabetes have at least a 10-fold greater risk of being hospitalized for soft tissue and bone infections of the foot

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Abbreviations: DFUs, Diabetic foot ulcers; MDRO, multidrug resistant organisms; MDR, multi drug resistance; PCR, polymerase chain reaction; MR, methyl red; NCBI, National Center for Biotechnology Information; TCA, trichloro acetic acid; IEF, isoelectric focusing; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; MIRSA, methicillin resistant *Staphylococcus aureus*; ESBL, extended-spectrum β -lactamase.

than the individuals without diabetes (Margolis et al., 2002). Approximately 50% of patients that fail antibiotic treatment will eventually require amputation.

Diabetic patients with foot ulcers have several factors that may be associated with a high risk of multidrug resistant micro-organisms (MDRM) carriage, such as inappropriate anti-biotic treatment, chronic course of the wound, reduced antibiotic concentration in the tissue and reduced antimicrobial effect in the wound environment and frequent hospital admission (Hartemann-Heurtier et al., 2004; Gadepalli et al., 2006; Ozer et al., 2010). The most important cause of antimicrobial resistance is overuse or inappropriate use of antibiotics (Margolis et al., 2002). It is also known that peripheral arterial diseases are often present in patients with DFUs and may lead to poor penetration of antibiotics into the lower limb tissues, thereby promoting growth of resistant bacterial strains. The widespread incidence of multi drug resistance (MDR) in both clinical and environmental settings is a potential threat to public health in most parts of the world (Saenz et al., 2004; Baker-Austin et al., 2008; Qing et al., 2012). Several reports suggest that inadequate selection and abuse of antimicrobials may lead to the development of resistance in various other bacteria and make the treatment of bacterial infections more difficult (Kolar et al., 2001).

It was a general belief that plasmids and transposons facilitate the spread of genetic material between species or genera of bacteria (Kolar et al., 2001; Harbottle et al., 2006). However in the 1980s, genetic elements integrons were identified on these mobile elements (Stokes and Hall, 1989). During the last few years the integron/gene cassette system has emerged as one of the best examples of capture and expression of new genes (Harbottle et al., 2006), and became a hot topic of research for understanding the mechanism of antibiotic resistance (Bzrlow and Pemberton, 2004). Integrons possess two conserved segments, the 5' CS and the 3' CS, separated by a variable region (VR), which include integrated antibiotic resistance genes (Qing et al., 2012). The 5' CS contains the *int* gene, a gene cassette insertion site, *attI* and, on the opposite strand, a common promoter region, P1-P2, directed towards the site of integration. Most inserted cassettes lack their own promoter and are expressed from the common promoter region. Integrons are classified according to the integrase sequence (El-Najjar et al., 2010).

Till now, five classes of integrons (classes 1, 2, 3, 4, 5) have been reported (Mazel, 2006). Of these, class 1 integrons are most common among multi-drug resistant Gram-negative genera including *Escherichia* (Saenz et al., 2004), *Klebsiella* (Girlich et al., 2000), *Salmonella* (Gebreyes and Altier, 2002), and *Serratia* species (Centron and Roy, 2002).

Polymerase chain reaction (PCR) amplification across highly conserved sequences has proved very useful and routine tool for screening and characterization of integrons from different bacteria (White et al., 2001; Chang et

et al., 2009). Although increasing incidence of antimicrobial resistance is becoming a serious problem in India, there is paucity of data dealing with the incidence of multiple-antibiotic resistance bacteria from DFUs. Additionally, little is known about the protein turn over in integron-possessing bacteria following growth with antibiotics. The aim of this study was to determine antibiotic susceptibility profile and screening of class 1 integron in bacteria isolated from DFUs. An attempt was also made to study alteration in protein profile (proteome) of a multiple-antibiotic resistant bacterium following growth with a combination of antibiotics.

MATERIALS AND METHODS

Patients

This study was conducted in the Department of Endocrinology and Metabolism, and Department of General Surgery, Sir Sunderlal Hospital, Institute of Medical Sciences, in collaboration with the School of Biotechnology, Banaras Hindu University, Varanasi. The study was conducted after seeking prior approval of the ethical committee of the Institute (Ref. No. Dean/2009-10/555 dated July 11, 2009). In total, 116 diabetic patients attending to the hospital between January 2010 and October 2011 were examined and four patients suffering from severe DFUs (grades IV and V) were selected for this study. Prior written consent was obtained from every recruited patient. Grading of DFUs that is, grade 0-hyperkeratosis; grade I -superficial ulcers; grade- II deep ulcers; grade- III tendonitis, osteomyelitis, cellulites, or abscess; grade- IV gangrene of a toe or forefoot; and grade -V massive gangrene of the whole foot was done according to Wagner (1981).

Sample collection

Initially, debridement was done with meticulous care to eliminate the colonizing bacteria from the ulcers. For sample collection, each DFU was cleaned with sterile saline and thereafter superficial swab sample was collected from the center of ulcer by applying a sterile cotton-tipped applicator. Tissue biopsy samples were obtained from the deep tissue of the ulcer using a sterilized punch biopsy needle (6 mm) under local anaesthesia. All specimens were transported by sterile containers; swabs in a tube containing sterile saline and biopsy samples in containers without medium. The specimens were examined quickly in the microbiology laboratory for Gram's staining.

Isolation and identification of bacteria

Each sample (swab and biopsy) was plated directly on different aerobic growth media such as 5% sheep blood agar, MacConkey agar, and chocolate agar and the plates were incubated at 35°C in an incubator. Biopsy samples were gently macerated before inoculation. The plates were examined after 24-36 h of incubation and distinct colonies appearing on each plate were picked up and restreaked on respective media. Isolates with distinct morphotypes from each plate were selected for further characterization. Tentative identification of different isolates was made on the basis of Gram's staining and morphological characters as well as biochemical tests namely, catalase, nitrate reductase, urease, Simmons citrate utilization and methyl red (MR) as per the standard methods. Identity of six isolates which carried multiple antibiotic resistance character was confirmed by 16S rDNA amplification and sequencing.

Amplification and sequencing of 16S rDNA

Genomic DNA was extracted by DNeasy Tissue Kit (Qiagen, Germany) according to the instructions of manufacturer. 16S rRNA gene (1.5 kb) was amplified by universal primer in a final volume of 50 μ l as described by Jha and Kumar (2009). Amplified products were subjected to electrophoresis in 1.0% agarose gel, stained with ethidium bromide and visualized under UV light in a gel documentation unit (BioRad Laboratories, USA). The PCR amplified fragment was purified from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen). Sequencing of amplified product was done on commercial basis from Chromous Biotech Pvt. Ltd. (Bangalore, India).

The sequences obtained were matched with the GenBank data base applying the algorithm BLASTn program to identify the most similar 16S rDNA (Altschul et al., 1997). The nucleotide sequences of the 16S rDNA of 6 bacterial species were submitted to National Center for Biotechnology Information (NCBI) GenBank and accession numbers have been obtained (Accession numbers, HQ163790, HQ163792, HQ163793, HQ163794, HQ163797, and HQ163798).

Antibiotic susceptibility test

Antimicrobial susceptibility test of different strains was done by the disc diffusion method using the Kirby-Bauer method (Bauer et al., 1966). Thirteen antibiotics; spectinomycin (100 μ g), streptomycin (20 μ g), trimethoprim (20 μ g), gentamicin (10 μ g), ampicillin (10 μ g), tetracycline (30 μ g), kanamycin (30 μ g), amikacin (30 μ g), augmentin (30 μ g), cefoperazone (75 μ g) meropenem (10 μ g), piperacillin-tazobactam (100/10 μ g) and clindamycin (2 μ g), were selected according to published recommendations and their widespread use in treatment of various diseases (Gadepalli et al., 2006). Ampicillin, penicillin, augmentin and clindamycin are penicillins, spectinomycin, streptomycin, gentamicin, kanamycin and amikacin are aminoglycosides, tetracycline belongs to tetracycline group, meropenem is a carbapenems, cefoperazone is a cephalosporin, and trimethoprim belongs to the group of chemotherapeutic agents. The disks were purchased from Micro Master Laboratories (Mumbai, India). Interpretation of result is based according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2007. Antibiotic susceptible, intermediately susceptible or resistant ability were assessed by measuring the diameter (mm) of the clear zone around the disc. Isolates showing resistance to more than two groups of antibiotics were designated as MDR.

Amplification of class 1 integron

The class 1 integron was amplified using forward primer 5'-GGC ATC CAA GCA GCA AG-3', and the reverse primer 3'-CS-5'-AAG CAG ACT TGA CCT GA-3' (Levesque et al., 1995), in a PTC-100 Thermal Cycler (MJ Research, Inc., USA). Reaction was performed in a final volume of 50 μ l, which included 1.5 U of *Taq* DNA polymerase, 1 X *Taq* assay buffer containing 1.5 mM MgCl₂, 125 μ mol of each dNTPs (Bangalore Genei, Bangalore), 50 pmol of each primers (Integrated DNA Technologies, USA) and 100 ng template DNA. Thermal cycle for the amplification was set at: initial denaturation at 94°C for 7 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 2 min 30 s followed by final extension at 72°C for 7 min. 5 μ l of the amplified PCR products were electrophoresed on a 1% agarose gel containing 0.5 μ g/ml ethidium bromide.

Two-dimensional gel electrophoresis

Protein extraction was done according to dos Santos et al. (2010).

Briefly, *E. coli* DF39TA cultures grown without antibiotics or with antibiotics namely tetracycline, kanamycin, spectinomycin and ampicillin (100 μ g/ml each) were harvested by centrifugation at 12000 rpm for 15 min at 4°C. The cell pellet was washed three times with MilliQ water and suspended in 2.5 ml of buffer [10mM Tris-Cl, 20 mM DTT and 1mM phenylmethylsulfonyl fluoride (Sigma, USA), pH-7.8] and passed through intermittent sonication (Branson Sonifier, USA) at maximum output and duty cycle for 2-3 min at 4°C. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. Cell extracts were treated with 1% SDS and subjected to trichloro acetic acid (TCA)-acetone precipitation. Precipitate was collected by centrifugation at 15000 rpm for 30 min at 4°C. The protein pellet obtained was washed twice with 90% acetone and thereafter air dried. To the dried pellet, 500 μ l of rehydration buffer [8M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer (GE Healthcare Ltd., UK) and 0.3% (w/v) DTT] were added and the suspension was kept at 4°C for 8-10h with intermittent vortexing to solubilize the protein pellet.

Isoelectric focusing (IEF) was carried out following the method of Gorg et al. (2000). Approximately 300 μ g of protein was applied on immobilized dry strip (pH 4-7, 13 cm, GE Healthcare Bio-Sciences AB, Uppsala) and rehydrated overnight at 20°C with rehydration buffer. Strips were focused on Ettan IPGphor3 system (GE Healthcare Ltd., UK) at 20°C using the following seven step program: a) 0-200 V for 4 h; b) 200-500 V for 2 h; c) 500-1000V for 2 h; d) 1000-2000V for 3 h; e) 2000-3500V for 2 h; f) 3500-5500 V for 2 h (steps a-f in linear mode), and g) 6500V constant until 60 kVh reached. The current limit was set at 50 μ A per strip. After IEF, each strip was incubated for 15 min in 10 ml of 50 mM Tris HCl buffer, pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% BPB, and 125 mM DTT, followed by a second incubation step in the same buffer, excluding DTT, which was replaced by 125 mM iodoacetamide. Proteins were separated in second dimension on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a vertical electrophoresis dual gel unit (Bangalore Genei, India) at constant current of 20 mA for 12 h and gels were stained with PlusOne Silver Staining Kit (GE Healthcare Ltd., UK) according to the instructions of manufacturer. 2-D gels were analysed using PDQuest 2-D analysis software (version 8.0.1) (BioRad Laboratories, USA). Images were analysed using stepwise spot detection and spot matching followed by differential expression analysis. Equal amount of protein was loaded in each gel and experiments were repeated at least three times.

Protein spot picking and mass spectroscopic analysis

Five distinct spots showing ca 4-fold increase in expression were excised from the gel by manual picking using sterile one touch spot picker into separate microcentrifuge tubes. Protein spots were sent to the Centre for Genomic Application (TCGA), New Delhi, India for matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopic analysis. The proteins were identified by comparing peptide mass fingerprints at the NCBI database using the Mascot search engine (<http://www.matrixscience.com>). The search parameters allowed for oxidation of methionines, carbamidomethylation of cysteines, one mis-cleavage of trypsin, and 30 ppm mass accuracy. The proteins were identified based on the first ranking result and Mascot scores >74, which indicated that the hits were significant.

RESULTS

Isolation and initial characterization of bacteria

23 strains of aerobic bacteria (10 from swabs and 13

Table 1. Isolation of multiple-antibiotic resistant bacteria from diabetic foot ulcers.

Patients ID	Grade of DFU*	Total types of bacterial species+	Multiple-antibiotic resistant isolates#
DF5	IV	<i>Escherichia coli</i> DF5SA	<i>Enterococcus</i> sp. DF5SB
		<i>Enterococcus</i> sp. DF5SB	
		<i>Klebsiella pneumoniae</i> DF5SC	
		<i>Staphylococcus haemolyticus</i> DF5TA	
		<i>Alcaligenes</i> sp. DF5TB	
		<i>Pseudomonas</i> sp. DF5TC	
DF18	IV	<i>Pseudomonas stutzeri</i> DF18SA	<i>Alcaligenes</i> sp. DF18SC
		<i>Alcaligenes</i> sp. DF18SC	
		<i>Escherichia coli</i> DF18TA	
		<i>Staphylococcus aureus</i> DF18TB	
		<i>Pseudomonas aeruginosa</i> DF18TC	
DF39	V	<i>Enterococcus</i> sp. DF39SA	<i>Escherichia coli</i> DF39TA
		<i>Alcaligenes</i> sp. DF39SB	
		<i>Pseudomonas stutzeri</i> DF39SC	
		<i>Escherichia coli</i> DF39TA	
		<i>Staphylococcus aureus</i> DF39TB	
		<i>Pseudomonas aeruginosa</i> DF39TC	
		<i>Proteus</i> sp. DF39TD	
DF36	IV	<i>Acinetobactor</i> sp. DF36SA	<i>Alcaligenes</i> sp. DF36TC
		<i>Enterococcus</i> sp. DF36SB	
		<i>Klebsiella pneumoniae</i> DF36TA	
		<i>Escherichia coli</i> DF36TB	
		<i>Alcaligenes</i> sp. DF36TC	

*, Grading of diabetic foot ulcer (DFU) was done according to Wagner's system; †, identification of isolates based on distinct morphological characters and biochemical tests; #, isolates showing resistance to more than three types of antibiotics were identified by 16S rDNA sequencing; DF, diabetic foot; S, swab; T, tissue; A-C, different isolates of tissue or swab.

from tissues) belonging to 8 genera and 11 species from DFUs of four patients were successfully isolated (Table 1). Of the 23 isolates, 20 (86.95 %) belonged to Gram-negative and 3 (13.04 %) to Gram-positive group. Two isolates namely *Escherichia coli* and *Alcaligenes* sp. were common in DFUs of all the four subjects whereas *Proteus* sp. and *Acinetobactor* sp were present in DFUs of DF39 and DF36 respectively. Interestingly, as many as 7 strains of bacteria were isolated from DFU of one patient (DF39). Occurrence of bacterial types was observed routinely higher in tissue (56.52%) than swab samples (43.47%).

Antibiotic sensitivity test

Antibiotic sensitivity test revealed that 16 isolates were resistant to either streptomycin and spectinomycin or trimethoprim. However, six isolates namely *Enterococcus* sp. DF5SB, *Staphylococcus haemolyticus* DF5TA, *Escherichia coli* DF39TA, *Alcaligenes* sp. DF18SC,

Pseudomonas sp. DF5TC, and *Alcaligenes* sp DF36TC showed resistance to more than three groups of antibiotics (Table 2).

Out of these, *Staphylococcus haemolyticus* DF5TA, *Pseudomonas* sp. DF5TC, *Enterococcus* sp. DF5SB and *Alcaligenes* sp. DF36TC showed resistance to four different groups of antibiotics. *Escherichia coli* DF39TA, and *Alcaligenes* sp. DF18SC, were found resistant to more than four groups of antibiotics. Based on antibiotics resistance profile, above six isolates could be designated as multidrug resistant bacteria (Table 2).

Identification of multiple drug resistance bacteria

All the bacterial isolates were identified on the basis of morphological characters and routine biochemical tests (Table 1). However, six isolates showing multiple antibiotics resistance character were identified by 16S rDNA (1.5kb) sequencing. Based on 16S rDNA (1.5kb) sequence homology, these isolates were identified as.

Table 2. Antibiotic resistance phenotype and size of class 1 integron of different isolates.

Isolate	Phenotype of resistance	Integron size (kb)
<i>Staphylococcus haemolyticus</i> DF5TA	Amp, Tmp, Tet, Aug, Mer	0.30
<i>Alcaligenes</i> sp. DF5TB	Spt, Str,	1.0
<i>Pseudomonas</i> sp. DF5TC	Spt, Str, Kan, Tmp, Gen, Aug, Cld, Mer	1.60
<i>Escherichia coli</i> DF5SA	-	-
<i>Enterococcus</i> sp. DF5SB	Spt, Str, Amp, Tet, Kan, Tmp, Gen, Aug	1.60
<i>Klebsiella pneumoniae</i> DF5SC	-	-
<i>Escherichia coli</i> DF18TA	Spt, Str	1.0
<i>Staphylococcus aureus</i> DF18TB	Amp, Tmp	0.72
<i>Pseudomonas stutzeri</i> DF18SA	-	-
<i>Alcaligenes</i> sp. DF18SB	Amp, Tmp	0.72
<i>Pseudomonas aeruginosa</i> DF18SC	Spt, Str, Kan, Tmp, Gen, Aug, Cef, Cld, Mer	1.20
<i>Escherichia coli</i> DF39TA	Spt, Str, Amp, Tet, Kan, Tmp, Gen, Aug, Cef, Cld, Mer	2.40
<i>Staphylococcus aureus</i> DF39TB	-	-
<i>Pseudomonas aeruginosa</i> DF39TC	Amp, Tmp	0.72
<i>Proteus</i> sp. DF39TD	Spt, Str, Amp	1.0
<i>Enterococcus</i> sp. DF39SA	-	0.85
<i>Alcaligenes</i> sp. DF39SB	Spt, Str	0.72
<i>Pseudomonas stutzeri</i> DF39SC	Amp, Tmp	0.72
<i>Klebsiella pneumoniae</i> DF36TA	-	-
<i>Escherichia coli</i> DF36TB	Spt, Str, Gen	1.60
<i>Alcaligenes</i> sp. DF36TC	Spt, Str, Kan, Tmp, Gen, Aug, Mer	0.85
<i>Acinetobacter</i> sp. DF36SA	-	-
<i>Enterococcus</i> sp. DF36SB	Spt, Str, Gen	1.60

Amp, Ampicillin; Amk, amikacin; Aug, augmentin; Cef, cefoperazone; Cld, clindamycin; Gen, gentamycin; Kan, kanamycin; Mer, meropenem; Pip/Taz, piperocillin/tazobactam; Spt, spectinomycin; Str, streptomycin; Tet, tetracycline and Tmp, trimethoprim.

Enterococcus sp DF5SB (HQ163798), *S. haemolyticus* DF5TA (HQ163797), *E. coli* DF39TA (HQ163793), *Alcaligenes* sp. DF18SC (HQ163792), *Pseudomonas* sp DF5TC (HQ163790), and *Alcaligenes* sp DF36TC (HQ163794), respectively.

Occurrence of class I integrons

Results of CS-PCR showed that 17 isolates harboured class 1 integron (variable region) with an amplicon size of ca. 0.3 to 2.4 kb (Table 2). It is evident from the result of Table 2 that five isolates had amplicon size of 0.72 kb, four had 1.60 kb, three contained 1.0 kb amplicon, two had 0.85 kb and the remaining three isolates had amplicon of 0.3, 1.20 and 2.4 kb size. Representation of amplified PCR product of class I integron of six isolates is presented in Figure 1.

Two-dimensional gel electrophoresis

Proteome analysis by 2-D gel electrophoresis of *E. coli* strain DF39TA grown with or without antibiotics showed distribution of protein spots in the region of pI 4-7. Figure

2 A-B depicts distribution of the protein spots of *E. coli* strain DF39TA grown without or with antibiotics (tetracycline, kanamycin, spectinomycin and ampicillin-100µg/ml each). Digital image analysis revealed the appearance of ca 200 and 266 spots in the untreated control and antibiotics-treated culture of *E. coli* strain DF39TA, respectively (Figure 2A-B). Of the 200 spots in the control culture, 126 spots matched with cultures grown with antibiotics. Analysis of spots of antibiotic-treated culture revealed that of the 126 spots, 62 were up regulated and 64 down regulated. It is also evident from the data of scatter diagram that 37 protein spots showed two-fold increase whereas 33 spots showed two-fold decrease in intensity following growth with antibiotics (Figure 2C). Several spots showed 3-4-fold increase or decrease in the intensity (Figure 2 D-E).

Five spots of interest (showing four-fold increase in intensity) were subjected to MALDI TOF MS for protein identification. Identity and other features of proteins are presented in Table 3. The five spots were identified as four unique proteins based on their possible function as OmpX, OmpA, OmpA-OmpF, Omp-tolC, and chaperone protein DnaK. Further analysis suggested that OmpX, OmpA, OmpA-OmpF, and Omp tolC are outer membrane

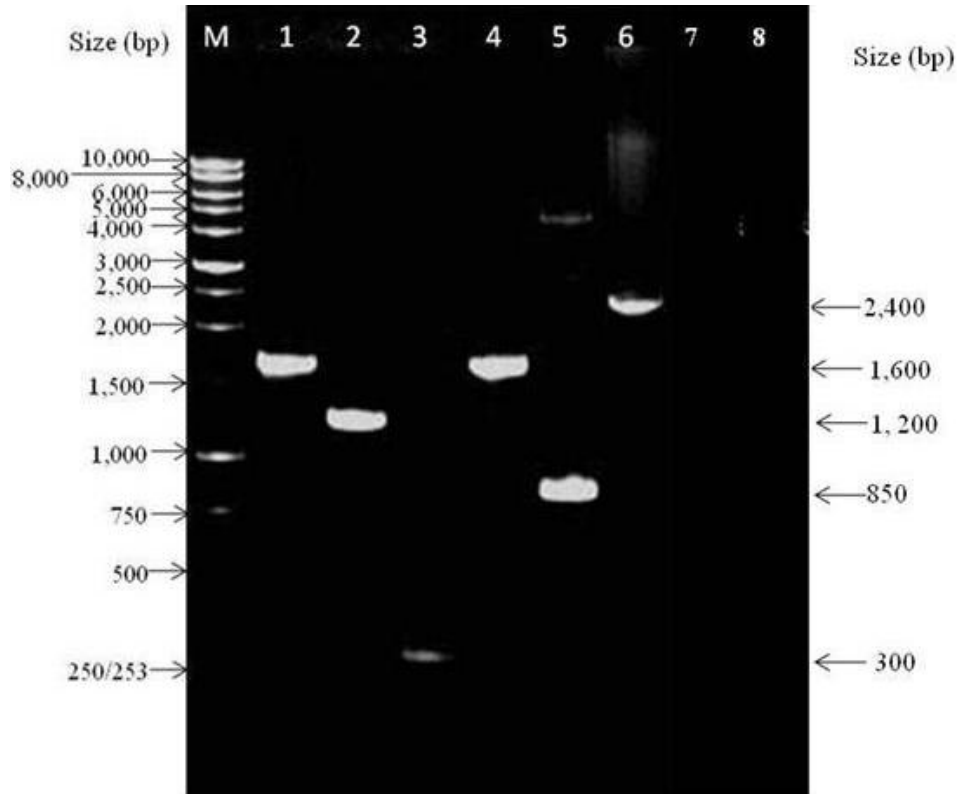


Figure 1. Polymerase chain reaction (PCR) amplification of class 1 integron with 5'CS-3'CS primers. Lane M, Molecular size marker (1 kb); lanes 1-8, various bacterial isolates; 1, DF5SB; 2, DF18SC; 3, DF5TA; 4,DF5TC; 5, DF36TC; 6, DF39TA; 7, negative control with *E. coli* JM109 template; and 8, negative control (without template).

(OM) proteins and chaperone DnaK is a cytoplasmic/inner membrane protein (Table 4).

DISCUSSION

The present study suggests that the prevalence of multi drug resistance organism (MDRO) is fairly common in severe DFUs and supports the finding of earlier studies (Hartemann-Heurtier et al., 2004; Gadepalli et al., 2006). Hartemann-Heurtier et al. (2004) reported that about one-third of patients with a history of previous hospitalization for the same wound, and 25% of patients with osteomyelitis, had MDRO-positive specimens. Gadepalli et al. (2006) also noted 44.7 and 56.0% ESBL-producing and methicillin resistant bacteria, respectively in DFUs from South India.

Incidence of high rate of antibiotic resistance in this study could be due to the fact that S.S. Hospital of Banaras Hindu University, Varanasi, is a tertiary care hospital with widespread usage of broad spectrum antibiotics leading to selective survival advantage of bacteria. Additionally, increase in antimicrobial resistance might be the result of irrational use of antibiotics and the transfer of

resistance genes by transport means including antibiotic resistant plasmids, bacterio-phages, transposons and integrons. Since a plasmid or transposon can carry several resistance indexes, simultaneous resistance to multiple antimicrobial agents may develop and the result would be MDROs. Although we could not estimate ESBL production in any isolates, the role of extended spectrum β -lactamase production in conferring resistance to antibiotics may not be ruled out. The prevalence of methicillin resistant *Staphylococcus aureus* (MIRSA) and extended-spectrum β -lactamase (ESBL)-producing Gram-negative bacteria in DFUs has been reported by other researchers (Hartemann-Heurtier et al., 2004; Gadepalli et al., 2006). An important finding of this study relates to the dominance of Gram-negative aerobic bacteria in DFUs. This is contrary to the findings of certain other workers who reported Gram-positive aerobes as predominant bacteria (Ozer et al., 2010).

Out of 16 isolates showing resistance to antibiotics, six isolates namely *E. coli* strain DF39TA, *S. haemolyticus* strain DF5TA, *Enterococcus* sp. DF5SB, *Pseudomonas* sp. DF5TC, *Alcaligenes* sp. DF18SC and *Alcaligenes* sp. DF36TC are of major concern as they elicited resistance to more than three groups of antibiotics.

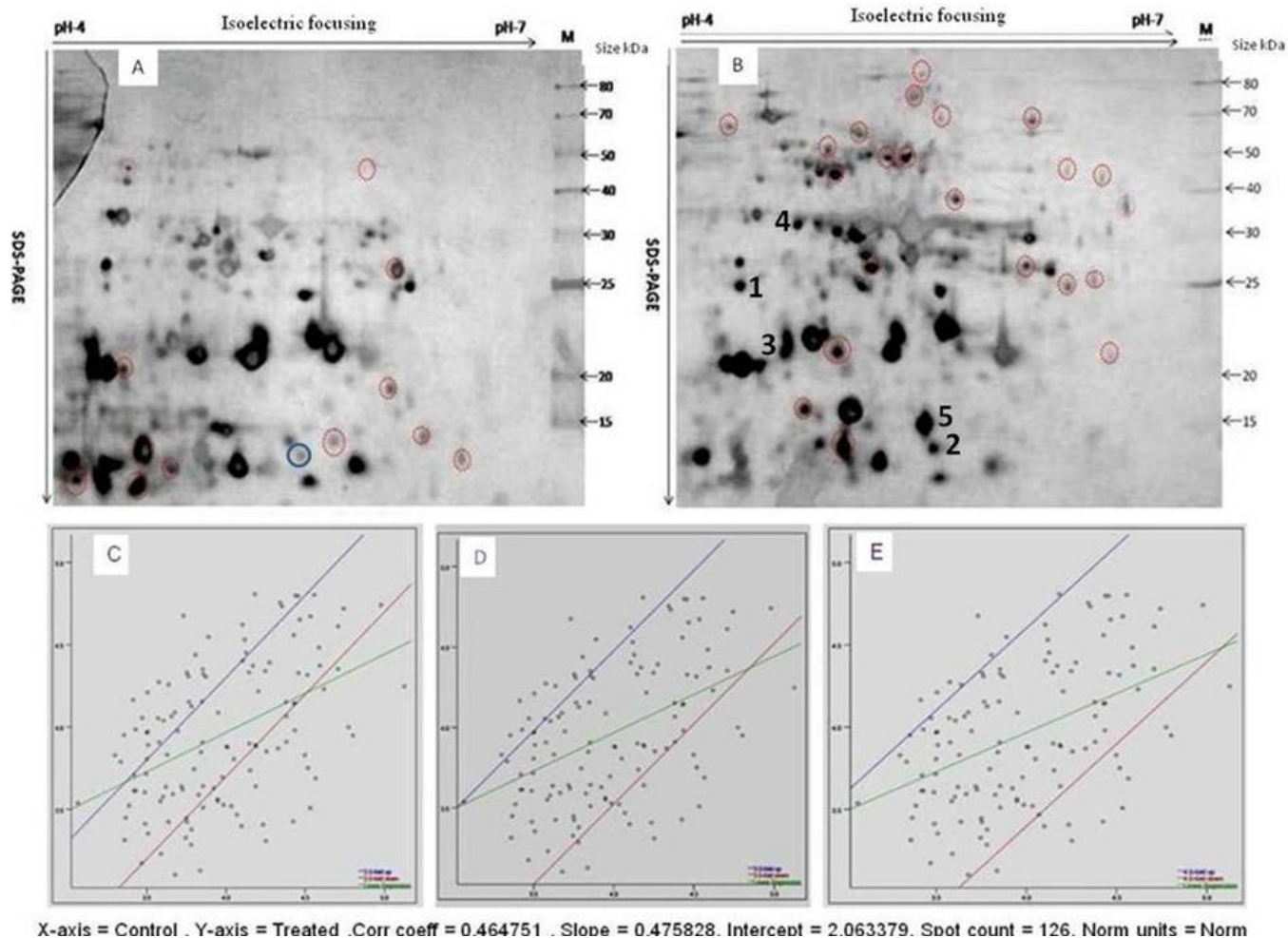


Figure 2. Two-dimensional gel images of protein spots of *E. coli* strain DF39TA. A, Control culture grown without antibiotic; B, *E. coli* strain DF39TA grown with four antibiotics namely ampicillin, kanamycin, tetracycline, and spectinomycin; C-E, scatter diagram of protein spots showing increase/decrease in intensity of spots: C, two-fold, D, three-fold, and E, four folds. X and Y axes represent spots of control and antibiotics-treated cultures, respectively.

Of the six isolates, *E. coli* strain DF39TA was resistant to 11 antibiotics belonging to six different groups. Findings of this study are close to that from non-pathogenic *E. coli* strains obtained from food products of animal origin and from fecal samples wherein multiple-antibiotic-resistant phenotypes to as many as 12 antibiotics of different groups were noted (Saenz et al., 2004). Additionally, clinical isolates namely *E. coli*, *S. haemolyticus*, *Enterococcus* sp., *Pseudomonas* sp., and *Alcaligenes* sp. have been reported to confer resistance to trimethoprim, streptomycin and spectinomycin (White et al., 2001; Lindstedt et al., 2003; Nogrady et al., 2005). All these reports suggest that infection with MDR strains may probably limit the choice of antibiotic treatment leading to longer duration of hospital stay and complicating the management of DFUs.

That the multiple-antibiotic-resistant phenotypes in various isolates could be due to the presence of class 1 integron of variable region (VR) is reinforced by the fact

that 17 isolates showed amplification of amplicons in the range of 0.3 to 2.4 kb size. Our finding is in agreement with the data of El-Najjar et al. (2010) who reported prevalence of class 1 integron from uropathogenic *E. coli* strains resistant to antibiotics. As majority of the isolates (n-11) in this study showed resistance to spectinomycin, streptomycin and/or trimethoprim, it appears that the presence of aminoglycoside resistance (*aadA*) and trimethoprim resistance (*dfrA*) determinants play important role in growth and survival of bacteria. Above conclusion is also supported from the studies conducted in Asia and Europe where high prevalence of the aminoglycoside resistance determinant (*aadA*) and trimethoprim resistance determinant (*dfrA*) was noted in several isolates (Saenz et al., 2004; Gadepalli et al., 2006; Chang et al., 2009; El-Najjar et al., 2010).

To our knowledge, occurrence of classes 1 and 2 integrons in clinical isolates of *Enterococcus* spp. was not known earlier and has been reported only recently (Xu et

Table 3. Identification of up-regulated protein of *E. coli* strain DF39TA after growth with different types of antibiotics*.

Spot no. ⁺	Protein	Gene ID	Protein accession No.	No. of amino acid	MW/ pI	Mascow score
1	OmpX	293403808	ZP_06648108.1	131	14759.2/ 5.75	200
2	OmpA	293409074	ZP_06652911.1	350	37479.1/ 5.78	587
3	OmpA-OmpF	293413461	ZP_06656886.1	350	37451.0/ 5.65	495
4	Omp tolC	293448943	ZP_06663796.1	490	53371.2/ 5.22	488
5	Chaperone protein DnaK	312965775	ZP_07780362.1	633	68522.3/ 4.81	395

*Culture was grown with tetracycline, kanamycin, spectinomycin and ampicillin (100µg/ml each); ⁺spots showing ca 4-fold up-regulation were selected for MALDI TOF MS analysis.

Table 4. Tentative function, localization, and gene ORF position of selected proteins of *E. coli* strain DF39TA after growth with antibiotics.

Protein	Gene ORF position	Trend in antibiotic treated vs. control	Biological process	Cellular location
OmpX	NZ_GG749217.1: 313566-313961	Four fold up regulated	Induced beta-lactam resistance	Outer membrane (OM)
OmpA	NZ_GG749328.1: 293351-294403	"	Stress survival	OM
OmpA-OmpF	NZ_GG749165.1: 897624-898676	"	Stress survival	OM
Omp tolC	NZ_GG749140.1: 467236-468708	"	Multidrug efflux and protein export	OM
Chaperone protein DnaK	NZ_ADUL01000057.1: 348814-350715	"	Stress Survival caused by antibiotics	Cytoplasm and inner membrane

al. 2010; Yan et al., 2010). Yan et al. (2010) were probably the first to report the presence of class 1 and 2 or both the integrons in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium*. Subsequently, Xu et al. (2010) also reported class 2 integron in clinical *E. faecalis* and class 1 integron in *E. faecium* from South China. Occurrence of integron in *Enterococcus* spp in our study is new addition and strengthen the findings of above report. However, detailed characterization of *Enterococcus* spp. integron is needed to find out resistance determinant present on this fragment.

Protein profile related to antibiotics resistance has been studied in a number of bacteria (Xu et al., 2006; Li et al., 2007; Roncada et al., 2009; dos Santos et al., 2010). However, little, if any, information is available pertaining to the changes in total protein profile of multiple antibiotic resistant bacteria from DFUs. Results of this study clearly suggest alteration both in qualitative and quantitative terms in proteome of *E. coli* strain DF39TA grown with or without antibiotics. Xu et al. (2006) employed proteomic approach to characterize functional outer membrane proteins of *E. coli* K-12 resistant to tetracycline and ampicillin. They identified outer membrane proteins such as TolC, OmpC and YhiU in this strain as a result of antibiotic resistance. Similarly, dos Santos et al. (2010) reported increase in abundance of 12 protein spots in *E. coli* resistant to piperacillin/tazobactam as compared to sensitive strain. Their study showed the role of a multidrug efflux pump system in *E. coli* resistance to piperacillin/tazobactam. We also noted more than four

fold increase in expression of OmpX, OmpA, OmpA-OmpF, Omp-tolC, and chaperone protein DnaK in *E. coli* strain DF39TA under stress of antibiotics. Furthermore all these proteins excluding chaperone protein DnaK were identified as outer membrane proteins. It is well documented that these proteins are responsible for antimicrobial resistance in different bacteria (dos Santos et al., 2010).

Prediction of function of these proteins in our analysis also suggested their roles in mitigating stress or in inducing beta lactam resistance or in multidrug efflux pump system. Altogether our findings pertaining to protein turn over during antibiotics stress are consistent with earlier reports and it may be safely concluded that changes in protein abundance may allow multiple drug resistant microorganisms to develop molecular changes in an effort towards adaptation to adverse environmental conditions.

Conclusions

In conclusion, data obtained reveal the occurrence of multiple-antibiotic resistant bacteria in diabetic foot ulcers and suggest the role of class 1 integron in conferring resistance to antibiotics. The gene cassettes present in integrons may explain the phenomenon of broad resistance of these bacteria to a number of antibiotics. Our data also reveal subtle changes in the proteome of a multiple antibiotic resistant strain (*E. coli* strain DF39TA)

suggesting that the misuse of antibiotics may also interfere with cell physiology which in turn may pose problem in the treatment of infectious diseases. Since the worldwide prevalence of antibiotic-resistant bacteria is on increase and may cause serious threat to human health, understanding the mechanism (s) of antibiotics resistance using both genomics and proteomics approaches could assist in developing strategies for better treatment.

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REFERENCES

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, 25:3389-3402.
- Baker-Austin C, McArthur JV, Tuckfield RC, Najarro M, Lindell AH, Gooch J, Stepanauskas R (2008). Antibiotic resistance in the shellfish pathogen *Vibrio parahaemolyticus* isolated from the coastal water and sediment of Georgia and South Carolina, USA. *J. Food Prot.* 71:2552- 2558.
- Bauer RW, Kirby MDK, Sherris JC, Turck M (1966). Antibiotic susceptibility testing by standard single disc diffusion method. *American J. Clin. Pathol.* 45:493- 496.
- Bzrlow RS, Pemberton JM (2004). Isolation and characterization of integron containing bacteria without antibiotic selection. *Antimicrob. Agents Chemother.* 48:838- 842.
- Centron D, Roy PH (2002). Presence of a group II intron in a multiresistant *Serratia marcescens* strain that harbors three integrons and a novel gene fusion. *Antimicrob. Agents Chemother.* 46:1402-1409.
- Chang YU, Fang YT, Tsai SM, Chang LL, Yu WL (2009). Characterization of class 1 integrons and gene cassettes in clinical isolates of *Klebsiella pneumoniae* from Taiwan. *Diag. Microbiol. Infect. Dis.* 65:214-216.
- Clinical and Laboratory Standards Institute (2007). Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement. M100-S17. Wayne, PA, USA: Clinical and Laboratory Standards Institute
- dos Santos KV, Diniz CG, Veloso Lde C, de Andrade HM, Giusta Mda S, Pires Sda F, Santos AV, Apolônio AC, de Carvalho MA, Farias Lde M (2010). Proteomic analysis of *Escherichia coli* with experimentally induced resistance to piperacillin/tazobactam. *Res. Microbiol.* 161:268-275.
- EI-Najjar NG, Farah MJ, Hashwa FA, Tokajian ST (2010). Antibiotic resistance patterns and sequencing of class I integron from uropathogenic *Escherichia coli* in Lebanon. *Lett. Appl. Microbiol.* 51:456-461.
- 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* 29:1727- 1732.
- Gebreyes WA, Altier C (2002). Molecular characterization of multidrug resistant *Salmonella enterica* serovar Typhimurium in swine. *J. Clin. Microbiol.* 40:2813- 2822.
- Girlich D, Karim A, Poirel L, Cavin MH, Verny C, Nordmann P (2000). Molecular epidemiology of an outbreak due to IRT-2 beta-lactamase-producing strains of *Klebsiella pneumoniae* in a geriatric department. *J. Antimicrob. Chemother.* 45:467-473.
- Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W (2000). The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* 21:1037-1053.
- Harbottle H, Thakur S, Zhao S, White DG (2006). Genetics of antimicrobial resistance. *Animal. Biotechnol.* 17:111- 124.
- Hartemann-Heurtier A, Robert J, Jacqueminet S, Ha Van, Golmard GJL, Jarlier V, Grimaldi A (2004). Diabetic foot ulcer and multidrug-resistant organisms: risk factors and impact. *Diabetic Med.* 21:710-715.
- Jha PN, Kumar A (2009). Characterization of novel plant growth promoting endophytic bacterium *Achromobacter xylosoxidans* from wheat plant. *Microbial. Ecol.* 58:179- 188.
- Kolar M, Urbanek K, Latal T (2001). Antibiotic selective pressure and development of bacterial resistance. *Int. J. Antimicrob. Agents* 17:357-363.
- Levesque C, Piche L, Larose C, Paul R (1995). PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob. Agents Chemother.* 39:185-191.
- Li H, Lin XM, Wang SY, Peng XX (2007). Identification and antibody-therapeutic targeting of chloramphenicol-resistant outer membrane proteins in *Escherichia coli*. *J. Proteome Res.* 6:3628- 3636.
- Lindstedt BA, Heir E, Nygard I, Kapperud G (2003). Characterization of class I integrons in clinical strains of *Salmonella enterica* sub sp. *enterica* serovars Typhimurium and *Enteritidis* from Norwegian hospitals. *J. Med. Microbiol.* 52:141- 149.
- Margolis DJ, Taylor LA, Hofstad O, Berlin JA (2002). Diabetic neuropathic foot ulcers. The association of wound size, wound duration and wound grade on healing. *Diabetes Care*, 25:1835-1839.
- Mazel D (2006). Integrons: agents of bacterial evolution. *Nat Rev Microbiol.* 4:608-20.
- Nogrady N, Gado ITA, Paszti J (2005). Antibiotic resistance and class 1 integron patterns of non-typhoidal human *Salmonella serotypes* isolated in Hungary in 2002 and 2003. *Int. J. Antimicrob. Agents* 26:126-132.
- Ozer B, Kalaci A, Semerci E, Duran N, Davul S, Yanat AN (2010). Infections and aerobic bacterial pathogens in diabetic foot. *Afr. J. Microbiol. Res.* 4:2153-2160.
- Qing Y, Wenxia S, Qingqing W, Xuejun C, Jianping L, Mingming Z (2012). Emergence of carbapenem-resistant *Enterobacter cloacae* carrying IMP-26 metallo-β lactamase in class 1 integron and TEM-104 extended-spectrum β- Lactamase in conjugative plasmid. *Afr. J. Microbiol. Res.* 6:3186-3191.
- Roncada P, Deriu F, Gaviraghi A, Martino PA, Bonizzi L. (2009). Proteomic study of antibiotic resistance in *Escherichia coli* strains. *Vet. Res. Commun.* 33:S157- S160.
- Saenz Y, Brinas L, Domínguez E, Ruiz J, Zarazaga M, Vila J, Torres C (2004). Mechanisms of resistance in multiple-antibiotic-resistant *Escherichia coli* strains of human, animal, and food origins. *Antimicrob. Agents Chemother.* 48:3996- 3901.
- Singh SK, Gupta K, Tiwari S, Shahi SK, Kumar S, Kumar A, Gupta, SK (2009). Detecting aerobic bacterial diversity in patients with diabetic foot wounds using ERIC-PCR: A preliminary communication. *Int. J. Lower Extrem. Wounds* 8:203- 209.
- Stokes HW, Hall RM (1989). A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol. Microbiol.* 3:1669-1683.
- Wagner FW (1981). The dysvascular foot: a system for diagnosis and treatment. *Foot Ankle* 2:64-122.
- White PA, McIver CJ, Rawlinson WD (2001). Integrons and gene cassettes in the Enterobacteriaceae. *Antimicrob. Agents Chemother.* 45:2658-2661.
- Xu C, Lin X, Ren H, Zhang Y, Wang S, Peng X (2006). Analysis of outer membrane proteome of *Escherichia coli* related to resistance to ampicillin and tetracycline. *Proteomics* 6:462-473.
- Xu Z, Li L, Shirtliff ME, Peters BM, Peng Y, Alam MJ, Yamasaki S, Shi L (2010). First report of class 2 integron in clinical *Enterococcus faecalis* and class 1 integron in *Enterococcus faecium* in South China. *Diagn Microbiol Infect Dis.* 68:315-317.
- Yan H, Li L, Zong M, Alam MJ, Shinoda S, Shi L (2010). Occurrence and characteristics of class 1 and class 2 integrons in clinical bacterial isolates from patients in south China. *J. Health Sci.* 56:442-450.