Detection of *Escherichia coli* and Associated β-Lactamases Genes from Diabetic Foot Ulcers by Multiplex PCR and Molecular Modeling and Docking of SHV-1, TEM-1, and OXA-1 β-Lactamases with Clindamycin and Piperacillin-Tazobactam

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Abstract

Diabetic foot ulcer (DFU) is a common and devastating complication in diabetes. Antimicrobial resistance mediated by extended-spectrum β-lactamases (ESBLs) production by bacteria is considered to be a major threat for foot amputation. The present study deals with the detection of *Escherichia coli* and the prevalence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>OXA</sub> genes directly from biopsy and swab of foot ulcers of diabetic patients. In total, 116 DFU patients were screened, of which 42 suffering with severe DFUs were selected for this study. Altogether 16 *E. coli* strains were successfully isolated from biopsy and/or swab samples of 15 (35.71%) patients. ESBL production was noted in 12 (75%) strains. Amplification of β-lactamase genes by multiplex PCR showed the presence of *bla*<sub>CTX-M</sub> like genes in 10 strains, *bla*<sub>TEM</sub> and *bla*<sub>OXA</sub> in 9 strains each, and *bla*<sub>SHV</sub> in 8 of the total 16 strains of *E. coli*. Out of the ten antibiotics tested, *E. coli* strains were found to be resistant to ampicillin (75%), cefoxitin (56.25%), cefazolin (50%), meropenem (37.5%), cefoperazone (25%), cefepime (31.25%), ceftazidime (56.25%), and cefotaxime (68.75%) but all showed sensitivity (100%) to clindamycin and piperacillin-tazobactam. 3D models of the most prevalent variants of β-lactamases namely TEM-1, SHV-1, OXA-1, and ESBL namely CTX-M-15 were predicted and docking was performed with clindamycin and piperacillin-tazobactam to reveal the molecular basis of drug sensitivity. Docking showed the best docking score with significant interactions, forming hydrogen bond, Van der Waals and polar level interaction with active site residues. Findings of this study may be routinely used in clinical diagnostics of *E. coli* and associated *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>OXA</sub> like genes.

Abstract

Diabetic foot ulcer (DFU) is a common and devastating complication in diabetes. Antimicrobial resistance mediated by extended-spectrum β-lactamases (ESBLs) production by bacteria is considered to be a major threat for foot amputation. The present study deals with the detection of *Escherichia coli* and the prevalence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>OXA</sub> genes directly from biopsy and swab of foot ulcers of diabetic patients. In total, 116 DFU patients were screened, of which 42 suffering with severe DFUs were selected for this study. Altogether 16 *E. coli* strains were successfully isolated from biopsy and/or swab samples of 15 (35.71%) patients. ESBL production was noted in 12 (75%) strains. Amplification of β-lactamase genes by multiplex PCR showed the presence of *bla*<sub>CTX-M</sub> like genes in 10 strains, *bla*<sub>TEM</sub> and *bla*<sub>OXA</sub> in 9 strains each, and *bla*<sub>SHV</sub> in 8 of the total 16 strains of *E. coli*. Out of the ten antibiotics tested, *E. coli* strains were found to be resistant to ampicillin (75%), cefoxitin (56.25%), cefazolin (50%), meropenem (37.5%), cefoperazone (25%), cefepime (31.25%), ceftazidime (56.25%), and cefotaxime (68.75%) but all showed sensitivity (100%) to clindamycin and piperacillin-tazobactam. 3D models of the most prevalent variants of β-lactamases namely TEM-1, SHV-1, OXA-1, and ESBL namely CTX-M-15 were predicted and docking was performed with clindamycin and piperacillin-tazobactam to reveal the molecular basis of drug sensitivity. Docking showed the best docking score with significant interactions, forming hydrogen bond, Van der Waals and polar level interaction with active site residues. Findings of this study may be routinely used in clinical diagnostics of *E. coli* and associated *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, and *bla*<sub>OXA</sub> like genes.

Introduction

Diabetic foot infections (DFIs) are common, often resulting in potentially devastating complications in diabetic patients. DFIs are associated with high morbidity and risk of lower extremity amputation [1]. Limb amputation has a major impact on the individual, not only in distorting the body structure, but also with regard to loss of productivity, increasing dependency, and costs of treating foot ulcers if patients require inpatient care [2]. Wound infection, delay in wound healing, neuropathy and ischemia in combination with a foot ulcer are the most common causes of diabetes-related amputations [3]. Patients with diabetes are more likely to lose a limb than those without diabetes and up to eighty-five percent of lower-limb amputations in patients with diabetes are preceded by infected foot ulceration [3]. To meaningfully describe and evaluate the severity of foot ulcer, several systems of classification are currently in use [4,5]. Wagner’s system of classification is routinely used in determining the surgical intervention to foot ulcer on admission. In more superficial infections which are classified according to Wagner (Wagner grades I-II), aerobic gram-positive bacteria are the predominant organisms. In deeper wounds (Wagner grades III-V), gram-negative bacteria are frequently found [6]. *Escherichia coli*, *Proteus* spp., *Pseudomonas* spp., *Staphylococcus aureus* and *Enterococcus* spp. are the most frequent pathogens contributing to progressive and widespread tissue destruction [7]. In the patients with DFI, there is a predominance of *E. coli* (24.20%) and antibiotic resistance is wide spread in this species [8]. The predominant mechanism of resistance to β-lactams in *E. coli* is production of extended-spectrum β-lactamases (ESBLs). Bacterial strains producing these enzymes inactivate the drugs by hydrolyzing the β-lactam ring [9]. ESBLs -producing bacteria are emerging as a worldwide clinical threat. In the early 1960s, *bla*<sub>TEM-1</sub> was the first plasmid-mediated β-lactamase gene in *E. coli* [10]. Subsequently, another common
β-lactamase gene bla_{SHV-1} was reported from *Klebsiella pneumoniae* and *E. coli*. Various new β-lactam antibiotics have been developed since 1960s for the treatment of patients which have resulted in emergence of other ESBL. Different types of β-lactamase have been reported during the 1990s however, TEM- and SHV-types are more common [11]. During the past decade, rapid and massive spread of CTX-M-type ESBLs have been reported. These enzymes are now the most prevalent ESBLs in Enterobacteriaceae and also occur rarely in *Pseudomonas* spp. and *Acinetobacter baumannii* [12] in Europe and in other parts of the world [13]. The list of ESBLs is increasing and the total number of well characterized ESBLs exceeds 200 [11].

The main objective of this study was to develop a simple and rapid method for the detection of *E. coli* isolates and associated β-lactamase genes (bla_{TEM}, bla_{SHV}, and bla_{OXA}) from patients suffering from DFI. Attempt was made to predict three dimensional (3D) model of TEM-1, SHV-1, OXA-1 (β-lactamases) and CTX-M-15 (ESBL). Furthermore, the identification of the amino acid residues crucial to the interaction between selected β-lactamases with clindamycin and piperacillin tazobactum was performed. Additionally, docking studies of TEM-1, SHV-1, OXA-1 and CTX-M-15 proteins with clindamycin and piperacillin-tazobactum were performed. It is anticipated that modeling and docking studies may be useful in developing new class of drugs to control ESBL-mediated antibiotic resistance problem in DFUs.

### Materials and Methods

#### Patients and Sample Collection

This study was conducted in the School of Biotechnology. Samples and details of patients were obtained from the Department of Endocrinology and Metabolism, and the Department of General Surgery, Sir Sunderlal Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi. Approval of the institutional ethics committee of Banaras Hindu University (Ref. No. Dean/2009-10/555 dated July 11, 2009) was obtained to conduct this study. Prior written consent was also obtained from every recruited patient. In total, 116 diabetic foot patients attending to the hospital between January 2010 and October 2011 were screened and 42 suffering with severe DFIs (Wagner's grade III-V) were selected for the study. Grading of DFUs was done according to Wagner [4].

Tissue samples from infected DFUs were obtained from the ulcer using a sterilized 6 mm punch biopsy needle under local anaesthesia. Two swab and tissue samples from each patient were collected by washing the wound with sterile physiological saline. One swab and tissue sample was used for detecting *E. coli* through *in vitro* culture, the second set of sample was used for detecting *E. coli* by PCR.

#### Isolation and Identification of *E. coli*

A direct smear was made from each sample (swab and biopsy) and plated directly onto MacConkey agar. The inoculated plates were immediately placed in an aerobic environment and incubated at 35°C for 24 h. The plates were examined after 24 h of incubation and distinct pink colonies that appeared on each plate were picked up and restreaked on respective media. Tentative identification of *E. coli* was made on the basis of Gram’s staining, morphological characteristics, and biochemical tests namely, catalase, urease, Simmons citrate utilization and MR (methyl red) as per the standard methods. *E. coli* JM109 (Promega, USA) was used as reference strain.

#### Isolation of Genomic DNA

Genomic DNA of swab and biopsy samples was extracted using a fast tissue PCR Kit (MBI Fermentas, USA). Genomic DNA from the laboratory-grown cultures was isolated using a DNaseasy tissue kit (Qiagen, Germany) according to the instructions of the manufacturer. Plasmid DNA from *E. coli* strains was isolated using a PureLink HiPure plasmid miniprep kit (Invitrogen, USA) according to the instructions of the manufacturer.

### Table 1. Details of resistance phenotypic and genetic characteristics of the *E. coli* strains.

| Serial No. | E. coli strains | Cefotaxime | Ceftazidime | ESBL DDSST | Etest for confirmation | ESBL |
|------------|----------------|------------|-------------|------------|-----------------------|------|
|            |                |            |             |            | CAZ/CLA | CTX/CTL | TEM | SHV | OXA | CTX-M |
| 1          | DF3SB          | S          | S           | –          | –        | –     | TEM-1 | SHV-12 | OXA-1 | CTX-M-9 |
| 2          | DF55C          | R          | S           | +          | –        | +     | –     | TEM-1 | –     | OXA-1 | CTX-M-15 |
| 3          | DF65A          | R          | R           | R          | +        | +     | +     | –     | –     | –     | CTX-M-15 |
| 4          | DF75A          | R          | R           | +          | +        | +     | –     | –     | –     | –     | CTX-M-15 |
| 5          | DF95B          | R          | R           | R          | +        | +     | –     | –     | –     | –     | CTX-M-15 |
| 6          | DF95TA         | R          | R           | +          | +        | +     | –     | –     | –     | –     | CTX-M-15 |
| 7          | DF13TB         | R          | S           | +          | +        | –     | –     | SHV-5 | –     | –     | CTX-M-15 |
| 8          | DF185A         | S          | S           | –          | –        | –     | –     | –     | SHV-1 | –     | CTX-M-15 |
| 9          | DF29TA         | R          | R           | +          | +        | –     | –     | –     | –     | –     | CTX-M-15 |
| 10         | DF10T8         | R          | S           | +          | +        | –     | –     | –     | –     | –     | CTX-M-15 |
| 11         | DF30TA         | R          | R           | +          | +        | +     | –     | –     | –     | –     | CTX-M-15 |
| 12         | DF30TD         | R          | R           | +          | +        | +     | –     | –     | –     | –     | CTX-M-15 |
| 13         | DF31TA         | S          | S           | –          | –        | –     | –     | –     | –     | –     | –     |
| 14         | DF40TA         | S          | R           | +          | +        | +     | –     | –     | –     | –     | –     |
| 15         | DF43TD         | S          | S           | –          | –        | –     | –     | –     | –     | –     | –     |
| 16         | DF49A          | R          | R           | +          | +        | –     | –     | –     | –     | –     | CTX-M-3 |

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Primer Designing, Amplification and Sequencing of *E. coli* Specific 16S rDNA

Primer3 (http://frodo.wi.mit.edu/) tool was used for designing *E. coli* gene specific primers from species-specific region (16S rRNA dimethyladenosine transferase). 16S rDNA (1467 bp) was amplified from the template DNA of the reference strain *E. coli* JM109, strains of *E. coli* isolated from DFUs, and biopsy/swab samples of DFUs. Amplification was performed in a final volume of 50 μl containing 1×PCR assay buffer with 1.5 mM MgCl₂, 25 pmol of each primers (Fd.5’-TGTGGGAACGGCGAGTCGGAATAC-3’ and Rev 5’GGGCGCAGGGGATGAAACTCAAC-3’) (Integrated DNA Technologies, USA), 250 μmol each of the dNTPs, 1U *Taq* DNA polymerase (Bangalore Genie, Banhalore) and 100 ng of template DNA. Conditions for PCR amplification were: initial denaturation for 10 min at 94°C, 30 cycles of 40 s at 94°C, 40 s at 60°C and 1 min at 72°C followed by final extension of 7 min at 72°C. 5 μl of the amplified PCR product was electrophoresed on a 2% agarose gel in Tris-borate-EDTA buffer (TBE) containing ethidium bromide (0.5 μg/ml) and monitored in gel documentation unit (BioRad Laboratories, USA). 16S rDNA (1467 bp) amplified from *E. coli* (isolated from DFU) was sequenced to confirm the identity and to confirm the specificity of primers. Additionally, the specificity of primer was confirmed using template DNA from other gram-negative bacteria viz., *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., and *Pseudomonas* spp. Based on sequence similarity, the representative isolate was identified as *E. coli* strain DF39TA. The sequence was submitted to NCBI database under accession number JX017293.

Antibiotic Susceptibility Testing

Antimicrobial susceptibility was done by the disc diffusion method using the Kirby-Bauer method [14]. Ten antibiotics
Table 2. Stereo-chemical properties using PDBSum.

| Proteins | % Residue in most favoured regions | % Residue in additional allowed regions | % Residue in generously allowed regions | % Residue in disallowed regions |
|----------|------------------------------------|----------------------------------------|----------------------------------------|-------------------------------|
| OXA-1    | 87.7                               | 11.0                                   | 0.7                                    | 0.0                           |
| SHV-1    | 95.2                               | 4.3                                    | 0.5                                    | 0.0                           |
| TEM-1    | 95.7                               | 3.8                                    | 0.5                                    | 0.0                           |
| CTX-M-15 | 95.3                               | 4.1                                    | 0.6                                    | 0.0                           |

Multiplex PCR and Sequencing of \(\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{OXA}},\) and 16S rRNA Genes of \(\text{E. coli}\)

Multiplex PCR was performed in a single tube with primers of \(\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{OXA}}\) and 16S rRNA genes. PCR assay was performed in a total volume of 50 \(\mu\)l which contained; 25 pmol of the primers of 16S rRNA (Fd 5'-TGTTGAGCCGCAAGTCGGGAATAC-3' and Rev 5'-GGGCCGAGGGATGAAACT-CAAC-3'), 10 pmol primers of each of the \(\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{OXA}}\) as described by Dallenne et al. [20], 200 \(\mu\)M each of the dNTPs, 1 U of Taq DNA polymerase, 1 XPCR assay buffer with 1.5 mM MgCl\(_2\) and 100 ng of template DNA or 5 \(\mu\)l of macerated biopsy samples. PCR conditions were used as described by Dallenne et al. [20]. PCR was run in a PTC-100 Thermal Cycler (MJ Research, Inc., USA). 5 \(\mu\)l of the amplified PCR product was used for electrophoresis and visualization was made as mentioned above. Multiplex PCR was also performed separately for \(\text{bla}_{\text{CTX-M}}\) Gp1, \(\text{bla}_{\text{CTX-M}}\) Gp2 and \(\text{bla}_{\text{CTX-M}}\) Gp9 genes as described previously [20].

Amplified product of \(\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{OXA}},\) and \(\text{bla}_{\text{CTX-M}}\) genes was purified by QiAquick gel extraction kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Sequencing and homology search of the amplified products were done on commercial basis from Chromous Biotech Pvt Ltd., Bangalore, India. After complete annotation, the sequences were submitted to NCBI database (http://www.ncbi.nlm.nih.gov/) and all accession numbers are shown in Table S1.

Gene Annotation and Similarity Search

Sequences of \(\text{bla}_{\text{TEM-1}}, \text{bla}_{\text{SHV-1}}, \text{bla}_{\text{OXA-1}},\) and \(\text{bla}_{\text{CTX-M-15}}\) genes from \(\text{E. coli}\) strain DF3917A were subjected to ORF scan (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to identify coding regions (exons). FGENESB was used to predict operons and genes in raw sequences [21]. The predicted putative protein sequences were subjected to protein functional analysis using INTERPROSCAN.
version 4.4 [22]. These protein sequences were used for homology search. Similar sequences from different species were retrieved and aligned using ClustalW [23]. Phylogenetic tree was constructed using the UPGMA method and tree was inferred by bootstrap phylogenetic inference using MEGA4 [24]. The conserved motifs present in these sequences were analyzed using BLOCKS and MEME (multiple EM for motif elicitation) software version 3.5.7 [25]. For motif analysis, the selection of maximum number of motifs was set to 10 with minimum width of 10 amino acids, while for genes a maximum number of motifs were set to 20 while other factors were default selections for putative proteins.

Retrieval of the Target Protein Sequence and Template Identification

Predicted putative protein sequences of blaTEM-1, blaSHV-1, blaOXA-1, and blaCTX-M-15 genes were used as target for homology modeling. Discovery studio 3.1 [26,27] was used for comparative homology modeling of TEM-1, SHV-1, OXA-1, and CTX-M-15 protein using template structures. PDB advance BLAST (http://www.rcsb.org/pdb/home/home.do) was applied for template identification to construct 3D models of the target proteins.

Figure 4. Interaction of OXA-1 with; (a) clindamycin, and (b) piperacillin-tazobactam.
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Model Refinement and Evaluation

Predicted 3D models of TEM-1, SHV-1, OXA-1, and CTX-M-15 proteins were used for evaluation and refinement. 3D models were subjected to energy minimization using the steepest descent technique to eliminate bad contacts between protein atoms. Computations were also carried out in vacuo with the GROMOS96 43B1 parameters with Swiss Pdb Viewer (http:// expasy.org/spdbv/) tool. The backbone conformation of the predicted model was inspected using the Phi/Psi Ramachandran plot of PDBsum database (http://www.ebi.ac.uk/pdbsum/) [28] and RAMPAGE server (http://mordred.bioc.cam.ac.uk/rapper/rampage.php) [29]. The SUPERPOSE server [30] (http://wishart.biology.ualberta.ca/SuperPose/) was employed to perform structural alignment between the target and template structure. ProSA and ERRAT servers (https://prosa.services.came.sbg.ac.at/prosa.php; http://nihserver.mbi.ucla.edu/ERRATv2/) were used for model quality evaluation. Evaluated and refined models were deposited to Protein Model DataBase (PMDB; http://mi.caspur.it/PMDB/).

Active Site Prediction and Docking

After obtaining the final model, the possible binding sites of TEM-1, SHV-1, OXA-1, and CTX-M-15 proteins were searched using Q-SiteFinder (http://bmbpcu36.leeds.ac.uk/qsitefinder/). Ten binding sites were obtained for TEM-1, SHV-1, OXA-1, and CTX-M-15. These binding sites were compared to the active site of the template to determine the residues forming the binding pocket. Clindamycin \( (C_{18}H_{33}ClN_2O_5S \{[2S,4R]-N-[2-chloro-1-[[2R,3R,4S,5R,6R]-3,4,5-trihydroxy-6-methyl sulfanyloxan-2-yl] propyl]-1-methyl-4 propylpyrrolidine-2-carboxamide} \), and piperacillin-tazobactam \( (C_{53}H_{39}N_9O_{12}S_2 \{[2R,5R,6R]-6-[[2-[4-ethyl-2,3-dioxopiperazine-1-carbon yl]amino]-2phenylacetyl]a-mino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid; \( (C_{28}H_{36}N_2O_{12}S_2 \{[2R,5R,6R]-6-[[2-[4-ethyl-2,3-dioxopiperazine-1-carbon yl]amino]-2phenylacetyl]a-mino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid} \) were used for docking study. These inhibitors were docked using LibDock module of Discovery studio 3.1. Docking calculations and interactions were analyzed using DS visualizer.

Figure 5. SHV-1 interaction with; (a) clindamycin, and (b) piperacillin-tazobactam. doi:10.1371/journal.pone.0068234.g005
Results and Discussion

Isolation and Identification of E. coli

Sixteen strains of E. coli were successfully isolated from biopsy/swab samples of 15 out of 42 patients (35.71%) admitted to the S.S Hospital, Varanasi. Two E. coli strains were isolated from DFU of one patient (DF30). Identity of E. coli strains was confirmed by morphological characteristics, biochemical tests and amplification of the E. coli specific 16S rDNA. Similar to our findings 32.07% of the DFU patients from south India were reported to carry infection of E. coli in DFUs [31]. Alavi et al. [32] also reported that E. coli (23.8%) was the most predominant gram-negative organisms in DFUs from patients of Iran.

One of the interesting outcomes of the study was use of species specific primers for the identification of bacteria from DFUs. All the strains of E. coli isolated from DFU showed amplification of the desired fragment of 16S rDNA (1476 bp) with E. coli specific primers in PCR assay. Interestingly, DNA isolated from 42 biopsy/swab samples also resulted in amplification of E. coli specific amplicon in 8 biopsy and 7 swab samples. However, template DNA isolated from other gram-negative bacteria did not show amplification of E. coli specific amplicon. As the PCR-based results matched with the laboratory-grown cultures, it is concluded that direct diagnosis of E. coli and/or other species of bacteria by PCR is possible directly from biopsy/swab samples. That these strains indeed belonged to E. coli also became evident from the sequencing of amplified product from a representative strain of E. coli. The sequences showed 99% similarity with sequences available in the NCBI for E. coli.

Antibiotic Resistance Profiles

Antibiotic sensitivity test revealed that all the sixteen E. coli strains of DFUs show high percentage of resistance to a number of antibiotics. Prevalence of resistance among the isolates was; ampicillin (75%), cefoxitin (56.25%), cefazolin (50%), meropenem (37.5%), cefoperazone (25%), cefepime (31.25%) ceftazidime (56.25%), and cefotaxime (68.75%). However, all the strains showed sensitivity (100%) to clindamycin and piperacillin/tazobactam. Similar to our findings, occurrence of multiple antibiotics resistance has been reported in several bacteria but only few reports are available for bacteria isolated from DFUs.

Figure 6. Interaction of TEM-1 with; (a) clindamycin, and (b) piperacillin-tazobactam.
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Table 3. Molecular docking calculation of OXA-1, SHV-1, TEM-1, and CTX-M-15 proteins with clindamycin and piperacillin-tazobactam.

| Proteins | Drug            | PMDB-ID   | Absolute Energy | Conf-No. | Relative Energy | LibDock Score |
|----------|-----------------|-----------|-----------------|----------|----------------|---------------|
| OXA-1    | Clindamycin     | PM0078676 | 40.9053         | 59       | 10.1825         | 117.997       |
|          | Piperacillin-tazobactam | PM0078677 | 55.519          | 2        | 1.006           | 153.322       |
| SHV-1    | Clindamycin     | PM0078678 | 49.0649         | 53       | 18.3421         | 124.517       |
|          | Piperacillin-tazobactam | PM0078679 | 58.9188         | 28       | 4.4058          | 155.258       |
| TEM-1    | Clindamycin     | PM0078680 | 37.8714         | 15       | 7.14862         | 84.4853       |
|          | Piperacillin-tazobactam | PM0078681 | 58.8299         | 27       | 4.31884         | 68.6161       |
| CTX-M-15 | Clindamycin     | PM0078866 | 41.0630         | 17       | 14.1256         | 122.556       |
|          | Piperacillin-tazobactam | PM0078867 | 57.1310         | 8        | 3.15691         | 165.589       |
ESBL and Carbapenemase Production in *E. coli*

Of the 16 *E. coli* isolates, 12 (75%) were ESBL producers according to the results of phenotypic tests DDST and E test (Table 1). ESBL-producing strains were found to be resistant to β-lactam antibiotics namely, ampicillin (88%), ceftepime (50%), cefoperazone (25%), ceftriaxone (33.33%), cefotaxime (58.33%), ceftazidime (73%), and ceftetaxime (91.66%). Additionally, six isolates (37.5%) showed resistance to meropenem which seems uncommon for *E. coli* species. Surprisingly, all these six isolates did not show the presence of carbapenemase by MHT. Recently, Shanmugam et al. [37] and Sahu et al. [38] have reported as high as 43.4% and 37% strains of *E. coli* resistant to meropenem respectively. With the available data, it is indeed hard to assign the exact mechanism of resistance to meropenem, it would be essential to confirm the presence of various types of carbapenemase genes employing PCR assay. This is the shortcoming of the present study and needs further investigation.

Available reports dealing with the prevalence of ESBL producers amongst various isolates of *E. coli* show marked variations [7,33–36]. In fact, the prevalence of ESBL producing *E. coli* isolates show significant differences among geographical locations within India [34], and other parts of world ranging from 0% in Iceland to less than 1% in Estonia, 41% in Romania, 16.8% in Iran [35], 25.2% in Tiruchirapali, South India [39], 0% in Iceland to less than 1% in Estonia, 41% in Romania, 16.8% in Turkey [40]. Gadepalli et al. [7] reported 54.5% *E. coli* isolates as ESBL producers in a tertiary care hospital in New Delhi. Detection of ESBL producing strains of *E. coli* is of vital importance as they are responsible for the spread of resistance among different bacteria. A combination of factors such as co-selection due to MDR phenotypes, virulence factors, mobile genetic elements, clonal spread of virulent strains and the acquisition of diverse ESBL-bearing plasmids may facilitate the spread of ESBL and other resistances [41].

Occurrence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA</sub>, and *bla*<sub>CTX-M</sub> Genes and Comparative Analysis of the ESBL Phenotype

Multiplex PCR assay was employed to detect the prevalence of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA</sub>, and *bla*<sub>CTX-M</sub> like genes as well as *E. coli* in a single PCR reaction. Typical representation of multiplex PCR for simultaneous amplification of *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>OXA</sub>, and 16S rRNA genes from 5 (2 biopsy and 3 swab) samples are shown in Figure 1. The amplified products were identical to those obtained with different ESBL tests and the multiplex PCR amplification of the different ESBLs. The overall phenotypic results obtained with different ESBL tests revealed some discrepancies due to different substrate profiles of the different ESBLs. The overall phenotypic results obtained with different ESBL tests and the multiplex PCR amplification assay were in good agreement (Table 1). Of the 16 *E. coli* isolates, 12 (75%) were ESBL producer as per the results of phenotypic test comprising DDST and E-test (Table 1). Of 12 ESBL producing strains, 10 showed ESBL phenotype due to the presence of either *bla*<sub>CTX-M</sub> (CTX-M-1, -3, -9, and -15), *bla*<sub>TEM</sub> (TEM-10, -20, and -52) and *bla*<sub>SHV</sub> (SHV-2, -5, and -12) genes. One strain (DF40TA) showed ESBL phenotype due to the presence of *bla*<sub>TEM-10</sub> and *bla*<sub>SHV-2</sub> ESBL genes (Table 1). *E. coli* strain DF10TB showed ESBL phenotype but ESBL gene was absent. ESBL positive phenotype of DF10TB strain may be due to the presence of other ESBL-types (CTX-M-15 like, CTX-M-25 like, PER, GES, VEB) or by spontaneous mutation in *ampC* gene that codes for an amidase and induces constitutive overproduction of the AmpC enzyme leading to increased resistance to ESBLs such as oxyminocephalosporins (cefoxatine, ceftriaxone and ceftazidine).

Gene Annotation, Similarity Search and Phylogenetic Analysis

Genes, *bla*<sub>OXA-1</sub> (Class D, Genbank Id: JX294482.1, Genpept Id: AFR79064.1), *bla*<sub>SHV-1</sub> (Class A, Genbank Id: JX294483.1, Genpept Id: AFR79065.1) *bla*<sub>TEM-1</sub> (Class A, Genbank Id: JX294484.1, Genpept Id: AFR79066.1) and *bla*<sub>CTX-M-15</sub> (Class A, Genbank Id: JX294480.1, Genpept Id: AFR79062.1) were used for alignment and phylogenetic classification with similar bacterial proteins. INTERPROSCAN study revealed that the SHV-1, TEM-1, and CTX-M-15 showed similarity with three domains namely, IPR000871, IPR012338 and IPR023650 of β-lactamase family. However, OXA-1 β-lactamases showed similarity with two domains viz. IPR001460 (penicillin-binding protein) and IPR012338 (β-lactamase domain). Using these identified proteins, BLAST (basic local alignment search tool) search was performed
to find homologous regions in the sequences of different bacterial species available in the biological database (NCBI). Multiple sequence alignment revealed highly conserved regions in blaOXA-1, blaSHV-1, blaTEM-1, and blaCTXM-15 genes (Figures S1a-S4a). Phylogenetic analysis among different species against these four genes showed closest neighbors with at least 99% similarity (Figures S1b-S4b). E. coli blaOXA-1 gene alignment indicated multilevel consensus sequence against different organisms, viz. Klebsiella oxytoca, Shigella flexneri, Klebsiella pneumoniae, Proteus sp., Pseudomonas aeruginosa, Salmonella enterica (Figure S1b) whereas blaOXA-1 gene alignment revealed multilevel consensus sequence with Klebsiella pneumoniae, Klebsiella oxytoca, Shigella dysenteriae, Enterococcus faecalis, Enterobacter asburiae (Figure S2b). Similarly, blaTEM-1 gene showed highest percentage of similarity with Klebsiella pneumoniae, Serratia marcescens, Acinetobacter baumanii, Morganella morgani, Enterobacter cloacae, Proteus mirabilis, and Pseudomonas aeroginosa species (Figure S3b). The alignment of blaCTXM-15 gene showed multilevel consensus sequence against Shigella boydii, Enterobacter gergoviae, Morganella morgani, Klebsiella pneumoniae, Citrobacter freundii, Shigella sonnei and Proteus mirabilis (Figure S4b). However, one multilevel consensus motif (RPDERFPMMSTFKVWLGAV) was present in the protein sequences of all the genes (Figure S5).

Homology Modeling of OXA-1, SHV-1, TEM-1, and CTX-M-15 Proteins
The homology modeling of OXA-1, SHV-1, TEM-1, and CTX-M-15 proteins was performed with Discovery studio 3.1 and is represented in Figure 2. Additionally, the 3D model of these proteins was constructed using the PDB BLAST for template identification. Analyses revealed that OXA-1 shares 99% similarity as well as positivity with PDB code: 1M6K of E. coli [50]. Similarly, SHV-1 showed 99% similarity and 100% positivity with the PDB code: 3D4F crystal structure of Klebsiella pneumonia [51]. TEM-1 showed 94% similarity and 94% positivity with PDB code: 1ERM of E. coli [52]. In the case of blaCTXM-15, there were 86% similarity and 93% positivity with PDB code: 2ZQ8 crystal structure of E. coli. Electrostatic energy of predicted OXA-1, SHV-1, TEM-1, and CTX-M-15 models were -5659.92, -7887.66, -9139.24, and -7430.49 kcal/mol respectively as per the analysis done by CHARMM force field of Discovery studio 3.1. Based on simulation study, it became evident that the predicted models are highly stable. Details of modeling and simulation results for OXA-1, SHV-1, TEM-1, and CTX-M-15 are available in Table S2.

Model Assessment (Refinement and Evaluation)
Stereochemical quality of the predicted protein structure was assessed using RAMPAGE and PDBsum. The Ramachandran plot study of OXA-1, SHV-1, TEM-1, and CTX-M-15 revealed that more than 90% of residues were in favoured regions having good stereochemical quality. Analysis of OXA-1, SHV-1, and CTX-M-15 revealed that 93.5, 98.1, and 98.9% residues occur in the favoured region respectively, with no residues present in the outlier region. TEM-1 showed 98.3% residues in favoured region and 0.4% residues in outlier region (Table S3). No residues were observed in the disallowed region. Further analysis based on PDBsum showed that the residues present in the most favoured regions for OXA-1, SHV-1, TEM-1, and CTX-M-15 were 87.7, 95.2, 95.7, and 93.5% respectively (Table 2). ERRAT score for the models of OXA-1, SHV-1, TEM-1, and CTX-M-15 was 91.15, 82.12, 92.30, and 92.35 respectively which are well within normal range for a high quality model. The best refined and validated structures of OXA-1, SHV-1, TEM-1, and CTX-M-15 were deposited in the PMDB database with PMDB-IDs; PM0078326, PM0078325, and PM0078327 respectively. The weighted root mean square deviation (RMSD) of the Ca trace between the template and the final refined model of OXA-1, SHV-1, TEM-1, and CTX-M-15 showed that the target and the template structures are closely similar at the backbone and at the CA tom level, yielding a significant Z-score. Resolution of the predicted OXA-1, SHV-1, TEM-1, and CTX-M-15 structure showed significant resolution of 2.363, 1.677, 2.021, and 1.62A respectively using RESPROX server. Structure quality estimation using PROSA showed significant Z-scores of -6.04, -5.27, -7.66, and -6.38 for OXA-1, SHV-1, TEM-1, and CTX-M-15 respectively as compared to the template Z-score. Structure quality estimation using QMEAN also resulted in significant Z-score (Table S4). The modeled structures of OXA-1, SHV-1, TEM-1, and CTX-M-15 revealed that each monomer belongs to the alpha-beta: 3-layer (a/a) sandwich DD-peptidase/b-lactamase super family (3.40.710.10) (Figure S6). Secondary element composition is also available in Table S5.

Active Site Prediction of OXA-1, SHV-1, TEM-1, and CTX-M-15 Proteins
Among the ten binding sites obtained from Q-Site finder (Figure 3), site 1 was found highly conserved in OXA-1, SHV-1, and CTX-M-15 and therefore selected as the active site for docking study with clindamycin and piperacillin-tazobactam. However, sites 3 and 7 were found highly conserved in TEM-1 and thus used for docking purpose. The results of multiple sequence alignment and active site prediction revealed that the residues in site 1 of OXA-1 (LYS177, GLU171, ARG103, VAL104, SER105, GLN106, MET107, TYR108, LEU109, GLY110, LYS111, THR112, THR113, PRO114, ARG115, MET116, LEU117, ARG118, VAL215) were similar to the active site of TEM-1 template structure. Similarly, residues in site 1 of SHV-1 (GLU103, ASN104, MET105, TYR106, LEU107, GLY118, LYS119, THR120 and PHE135) were identical to the reported active site of E. coli template structure. All the 10 possible active sites for OXA-1, SHV-1, TEM-1, and CTX-M-15 were similar to the active site of K. pneumoniae template [51]. In the case of CTX-M-15, residues in site 1 (ARG120, VAL121, GLY122, LEU123, ILE124, PHE125, MET126, THR127, THR128, THR129, PRO130, MET131, ALA132, LEU133, ARG134, GLY135, ALA136, ILE137, and VAL138) were similar to the active site of K. pneumoniae template [51]. Among the 10 possible active sites for OXA-1, SHV-1, TEM-1, and CTX-M-15 models are available in Table S6. All these putative active sites show close similarity with the reported active sites of b-lactamases [51–53] and support our findings.

Molecular Docking of OXA-1, SHV-1, TEM-1, and CTX-M-15 Proteins
Docking of OXA-1, SHV-1, TEM-1, and CTX-M-15 protein was performed with clindamycin and piperacillin-tazobactam. Final docked conformations obtained for these inhibitors were evaluated considering the number of hydrogen bonds formed and the bond distance between atomic co-ordinates of the active site and inhibitor.
With OXA-1 it was noted that clindamycin and piperacillin-tazobactam interacted with the major active site cavity, with a site volume of 234 cubic Å. The residues namely, VAL 13, GLY 14, Tyr 15, Met 18, Arg 19, Met 20, Thr 21, Gly 23, Glu 24, and Gly 26 were mainly involved in the interaction with clindamycin (Figure 4a) whereas piperacillin-tazobactam interacted with Met 19, Tyr 28, Leu 31, Gly 32, Thr 33, Arg 34, Thr 36, and Glu 39 and were found as prominent active sites.

In both the complexes Ala 53, Ser 56, Arg 57, Gln 62, Ile 116, Lys 119, Leu 122, Ala 124, Phe 125, Leu 126, Asn 128, Met 129 and Thr 120 residues are commonly associated ESBL-producing strains isolated from DFUs patients. In view of high prevalence of blaCTX-M-15 like gene, it is recommended that multiplex PCR may be routinely used for the screening of this gene in ESBL-producing bacteria. Our findings also showed that majority of the ESBL-producing strains were resistant to β-lactams but showed 100% sensitivity to clindamycin and piperacillin-tazobactam. Furthermore, the 3D models of most prevalent variants of β-lactamases viz., TEM-1, SHV-1, OXA-1, and ESBL-like CTX-M-15 were predicted and docking studies with clindamycin and piperacillin-tazobactam were performed. The docking scores of TEM-1, SHV-1, OXA-1, and CTX-M-15 proteins with clindamycin and piperacillin-tazobactam showed significant interaction with active binding residues. The docking studies also revealed that Tyr 48, Glu 69, Ser 70, Glu 79, and Thr 120 of OXA-1; Thr 40, Arg 212, and Ile 214 of SHV-1; Ser 56 and Leu 172 residues of TEM-1 protein; Pro 163 residue of CTX-M-15 form hydrogen bonds with the side chain along with main chain interaction with above drugs. The results of the present study may provide useful insights for developing new antibiotic drugs to minimize ESBLs-mediated resistance problem of bacteria in DFU of diabetic patients.

Supporting Information

Figure S1 Phylogenetic analysis of blaoxa-1 like gene based on sequences obtained from NCBI database. (a) Multiple sequence alignment of blaoxa-1 gene sequence of E. coli DF39TA with other species and, (b)-phylogenetic tree showing similarity of E. coli DF39TA blaoxa-1 like gene sequence with closely related species. (DOC)

Figure S2 Phylogenetic analysis of blashv-1 like gene based on sequences obtained from NCBI database. (a) Alignment of blashv-1 gene sequence of E. coli DF39TA with other species, and, (b)-phylogenetic tree showing similarity of E. coli DF39TA blashv-1 like gene sequence with other species. (DOC)

Figure S3 Phylogenetic analysis of blatem-1 like gene based on sequences obtained from NCBI database. (a) Alignment of blatem-1 gene sequence of E. coli DF39TA with other species, and, (b)-construction of phylogenetic tree showing relatedness of E. coli DF39TA blatem-1 like gene sequence with other species. (DOC)

Figure S4 Phylogenetic analysis of blactx-m-15 like gene based on sequences obtained from NCBI database. (a) Alignment of blctx-m-15 gene sequence of E. coli DF39TA with other species, and, (b)-phylogenetic tree showing relationship of E. coli DF39TA blctx-m-15 like gene sequence with other species. (DOC)

Figure S5 Motif elucidation based on multilevel consensus sequence. (a)- Representative motif elucidation of OXA-1, SHV-1, TEM-1, and CTX-M-15 proteins, and (b)-multilevel consensus sequences for the MEME-defined motifs observed in OXA-1, SHV-1, TEM-1, and CTX-M-15 proteins from E. coli DF39TA. (DOC)
Figure S6  Secondary structure of various proteins. (a) - OXA-1, (b) - SHV-1, (c) - TEM-1, and (d) - CTX-M-15 proteins. (e) - represents key.

Table S1  Details of accession number of variants of blaTEM, blaSHV, blaCTX-M, and blaOXA genes of E. coli strains.

Table S2  Modeling and simulation details of OXA-1, SHV-1, TEM-1, and CTX-M-15 proteins.

Table S3  Stereo-chemical details of predicted models of OXA-1, SHV-1, TEM-1, and CTX-M-15 proteins using RAMPAGE.

Table S4  Model quality estimation of OXA-1, SHV-1, TEM-1, and CTX-M-15 proteins using QMEAN (http://swissmodel.expasy.org/qmean/cgi/index.cgi).

Table S5  Secondary compositional study using VADAR

Table S6  Ten predicted active binding sites for OXA-1, SHV-1, TEM-1, and CTX-M-15 using Q-SiteFinder.

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Author Contributions

Conceived and designed the experiments: AK SKS. Performed the experiments: SKS. Analyzed the data: SKS VKS. Contributed reagents/materials/analysis tools: AK. Wrote the paper: SKS AK.

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Table S2  Modeling and simulation details of OXA-1, SHV-1, TEM-1, and CTX-M-15 proteins.

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Author Contributions

Conceived and designed the experiments: AK SKS. Performed the experiments: SKS. Analyzed the data: SKS VKS. Contributed reagents/materials/analysis tools: AK. Wrote the paper: SKS AK.
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