3. MATERIALS AND METHODS

3.1. Study Design.
This was a prospective cohort study hospital based. Out of 469 diabetic patients (Type 1DM= 87, type 2 DM=382) admitted in the ward of Rajiv Gandhi Centre for Diabetes and Endocrinology, Jawaharlal Nehru Medical College Hospital of Aligarh Muslim University, Aligarh, India, during December 2008 to March 2011. 162 (Type1 DM=28, Type 2 DM=134) out of 194 diabetic foot ulcer patients who had infection were included in this study. All the subjects gave informed consent and the study was approved by the Institutional Ethics Committee (IEC), Bio-Ethical Committee (BEC) of the faculty of Medicine and the Committee of Advanced Studies and Research (CASR).

The diagnosis of diabetes mellitus was made in accordance with the guidelines laid-down by American Diabetes Association - 2008 (Diabetes 2008)

- FPG 126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.
- 2-h plasma glucose 200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
- In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose 200 mg/dl (11.1 mmol/l).

3.2. Clinical Examination.
A detailed history and physical examination was carried out for every subject. Age, Sex, anthropometric measurements (body mass index), duration of diabetes, glycemic control prior to and during the hospital stay (Prerna et al., 2011), lipid profile, presence of retinopathy (assessed by ophthalmologist and the stage of diabetic retinopathy was graded as: Within Normal Limits (WNL), Non-Proliferative Diabetic Retinopathy (NPDR), Preproliferative Diabetic Retinopathy (PPDR) and Proliferative Diabetic Retinopathy (PDR) according to International Clinical Diabetic Retinopathy (ICDR) Disease Severity Scale (Klein et al., 1989)), nephropathy (creatinine >1.5 mg% or presence of micro or macro-albuminuria), CCr was calculated according to MDRD
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formula [Levey et al., 1999]. Because of our relatively small sample size (n = 162), categories of 0 and 1 (no nephropathy or micro-albuminuria only; CCr ≥ 90 ml/min/1.73 m2; Category A), 2 and 3 (mild to moderate reduced CCr, CCr: 30-89 ml/min/1.73 m2; Category B), and 4 and 5 (severe reduced CCr or need for renal replacement treatment, CCr < 30 ml/min/1.73 m2; Category C) were combined, neuropathy (absence of perception of the Semmes-Weinstein monofilament at 2 of 10 standard planter sites on either foot) (Mayfield & Sugarman, 2000), peripheral vascular disease (ischaemic symptoms and intermittent claudication of rest pain, with or without absence of pedal pulses or posterior tibial pulses). The ankle-brachial index (ABI) was calculated as the ratio of the ankle systolic blood pressure (defined as the higher of the dorsalis pedis or posterior tibialis measurement,) divided by the higher brachial systolic pressure. Subject was classified as having PAD when they had an ABI ≤ 0.9 and/or when they had undergone a peripheral arterial bypass or amputation (Hartemann-Heurtier et al., 2004). ADA criteria for hypertension was used (systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg in subjects who are not taking antihypertensive medication or antihypertensive treatment yet present on admission). Hypercholesterolemia was defined as total serum cholesterol ≥150 mg/dL and hypertriglyceridemia as total serum triglyceride ≥200 mg/dL on the basis of ADA-2010 criteria. Coronary artery disease was determined on the basis of history of physician-diagnosed angina, myocardial infarction, or any previous revascularization procedure assessed by questionnaire. Cerebrovascular disease (TIA/ischaemic stroke) was assessed by history, specific neurological examination executed by specialists, and hospitalized or radiological (brain computed tomography or brain magnetic resonance) records of definitive TIA or stroke. All patients had blood pressure, serum glucose, creatinine, serum cholesterol levels, serum triglyceride levels, and urinary albumin excretion (UAE) values measured on first day of admission to the hospital. Duration of ulcer, site, and size of ulcer, history of smoking, history of previous amputation and clinical outcome were noted in every patient. Clinical assessment for signs of infection (swelling, exudates, surrounding cellulitis, odor, tissue necrosis, crepitation and pyrexia) was made by one researcher classifying the ulcers and determining the presence of clinical signs of infection. Ulcer size was determined by multiplying the longest and the widest diameters and expressed in centimeters square. The wound was
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graded and staged at the time of hospitalization according to the University of Texas Wound classification system as grade 1 (superficial wound, not involving, tendon, capsule or bone), grade 2 (wound penetrating to tendon or capsule) and grade 3 (wound penetrating bone or joint). Grade 0 patients (pre- or post-ulcerative site that has healed) were excluded from the study, and also with Meggit Wagner Wound Grading System (Wagner, 1981) as Grade 1(Superficial lesion), Grade 2 (deep ulcer to tendon or joint capsule), Grade 3 (deep ulcer with abscess, osteomyelitis, or joint sepsis), Grade 4 (Partial gangrene), and Grade 5 (Gangrene of entire foot). Grade 0 patients (no open lesion) were excluded from the study. Diagnosis of extension to the bone was made in majority of patients by probing with a sterile steel probe. In the absence of sinus tract or an exposed bone, a standard radiograph showing signs of osteomyelitis in the bone was considered definitive and later on MRI was done to confirm the osteomyelitis in suspected patients. Amputation was defined as the complete loss in the transverse anatomical plane of any part of the lower limb (Hartemann-Heurtier et al., 2004).

3.3. Clinical Investigations.

3.3.1. Collection of samples.

Blood samples were collected for the clinical investigations, between 8 to 10 am after an overnight fast of 10-12 hours for all the routine investigation. For urine, first morning urine sample was collected for routine urine examination.

- Blood samples were collected:
  - In EDTA Na vials for HbA1c,
  - In sodium fluoride vials for plasma glucose,
  - In plain vials for serum lipids and lipoproteins.

3.3.2. Plasma Glucose (glucose oxidase method).

**Principle:** Glucose oxidase catalyses the oxidation of alpha-D-glucose to D-glucono-1, 5 lactone (gluconic acid) with the formation of hydrogen peroxides. The oxygen liberated from hydrogen peroxide by peroxidase reacts with the O-dianisidine and oxidizes it to a red chromophore product. Estimation of glucose by glucose oxidase gives the true glucose concentration eliminating the interference by other reducing sugars.
3.3.3. Glycohaemoglobin [HbA1c].

**Principal:** The D-10 Hemoglobin A1c Program utilizes principles of ion-exchange high-performance liquid chromatography (HPLC). The D-10 delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured. The assay procedure was followed as per directives of the kit, Bio–Rad Laboratories Inc., Hercules, California, USA.

3.3.4: Serum Lipids.
The serum Lipid was estimated by the assay method as per directive of the kit: Ericokets, Ranbaxy diagnostics and cholesterol reagent by Point Scientific Inc. Michigan USA.

3.3.4.1: Serum Cholesterol.

**Principle:** Total cholesterol in the serum was determined by the cholesterol esterase oxidase peroxidase methods based. The intensity of the red colour produced is directly proportional to the total cholesterol in the sample when read at 520 nm. The assay procedure was followed as per the directives of Pointe Scientific, Michigan, USA.

3.3.4.2: Serum Triglycerides.

**Principle:** They were estimated by modified enzymatic method using colour reaction to produce a fast, linear, end point reaction. The intensity of the colour produced is directly proportional to the concentration of triglycerides in the sample when measured at 540 nm. The assay procedures were performed as per the directives of the Pointe Scientific Inc. Michigan, USA.

3.3.4.3: HDL – Cholesterol.

**Principle:** HDL-cholesterol was estimated on the principle that chylomicrons, very low density lipoproteins (VLDL), and low density lipoproteins (LDL) of serum are precipitated using buffered polyethylene glycol (PEP-6000). After centrifugation, High Density lipoprotein (HDL) remains in the supernatant. The cholesterol in HDL fraction
is then estimated by the enzymatic method using cholesterol esterase; cholesterol oxidase, peroxidase, 4-amino antipyrine and phenol. The assay procedures were performed according to the directives of kit: Ranbaxy Diagnostic division, New Delhi.

3.3.4.4: LDL- Cholesterol.
**Principle:** Cholesterol was calculated using Freidwald and Fredrickson's formula:

$$\text{LDL-C} = \text{Total Cholesterol} - \left\{ \frac{\text{Triglycerides}}{5} + \text{HDL-Cholesterol} \right\}$$

3.3.4.5: Urine Examination for Albumin.
Urinary albumin was quantitatively determined by immunotech albumin RIA kit and the assay procedures were followed as per directives of the kit i.e. Immunotech Company Prague, Czech Republic.

3.4. Microbiological Methods.

3.4.1. Collection & Transport of Samples.
The specimens were obtained at the time of admission in the ward using standard microbiological techniques for aerobic bacteria, anaerobic bacteria and fungal organisms.

3.4.1.1. Aerobic Bacteria.
The specimens were obtained at the time of admission by scraping the base of ulcer or the deep portion of the wound edge with a sterile transport swab with Amies medium without charcoal purchased from HiMedia (Product no MS684-50NO, HiMedia Mumbai India). The samples were obtained in duplicate and transported to the laboratory within 20 minutes of its collection [Gadepalli et al., 2006].

3.4.1.2. Anaerobic Bacteria.
With all aseptic precautions, pus for anaerobic culture was aspirated with the help of sterile syringe after the skin overlying the area was disinfected with a mixture of anaerobic disinfectant containing iodophor solution and 70% alcohol. If no pus was aspirated out, 2 to 3 ml of sterile saline was then injected and aspirated out for cultures.
One portion was immediately transferred into Robertson cooked meat medium (RCM) and then the air was expelled out from the syringe and needle was capped with a rubber stopper. This was then transported to the laboratory for direct microscopy and aerobic bacterial culture [Bailey & Scotts, 2007, Mackie & McCartney, 2007].

3.4.1.3. Fungal organisms
The specimens were obtained at the time of admission by scraping the base of ulcer or the deep portion of the wound edge with a sterile swab. The samples were obtained in duplicate and were transported to the laboratory within 20 minutes of its collection [Chellan et al., 2010].

3.5. Processing of Samples.
3.5.1. Direct Microscopy.
Smears were made from the specimen obtained from DFU and were stained with Gram's stain to study the presence of gram positive and gram negative cocci/bacilli. For fungal organisms, a 10% KOH mount and Lacto-phenol Cotton Blue (LCB) mount was made. The smears were also screened for the presence of inflammatory cells.

3.5.2. Culture and Identification.
The samples were cultured for aerobic bacteria, anaerobic bacteria and fungal organisms.

3.5.2.1. Aerobic Bacterial Culture.
Samples were inoculated on the blood agar plate (BA), Teepol Lactose Agar (TLA), Nutrient agar (NA) and simultaneously in brain heart infusion (BHI) broth and were incubated at 37°C for 18 hours. After the incubation period is over, on the basis of cultural characteristics, colony morphology on culture plates and biochemical reactions as per the standard methods, the organisms were identified (Mackie & McCartney 2007).

3.5.2.2. Anaerobic Bacterial Culture.
All aspirated pus samples were immediately transferred into RCM culture medium and were incubated for 40 to 48 hours at 35°C. After the incubation period is over,
approximately 0.3 mL was aspirated out using sterile syring and plated onto pre-reduced anaerobic agar plate (Hi-Media, Mumbai) and was incubated at 35°C in an anaerobic Jar (Hi-Media, Mumbai) with anaerobic gas pack (Hi-Media, Mumbai). Anaerobic bacteria were identified on the basis of cultural characteristics, colony morphology and biochemical reactions (Bailey & Scotts, 2007, Mackie & McCartney, 2007).

3.5.2.3. Fungal Culture.
The culture was done on two sabouraud dextrose agar (SDA) slants containing chloramphenicol (0.05 mg/ml) by rolling over the surface and subsequently in BHI broth also. One tube was incubated at 25°C and the remaining tube and BHI broth were incubated at 37°C. After initial inoculation and incubation, tubes were examined daily for fungal growth upto 3 weeks. The identification of fungal isolates was done as per standard techniques (Mackie & McCartney 2007).

(a). Identification for Yeast Fungi.
The identification of yeast fungi was in accordance to i) colony characteristics, ii) Germ tube test (GTT test), iii) growth at 42°C, iv) morphology on CMA, v) Sugar fermentation tests and vii) Sugar assimilation tests.

i. Colony characteristics:
The colony characters of the various yeasts were noted with respect to colony morphology; colour, texture, presence/absence of mycelial halo etc.

ii. Germ tube test (GTT):
The fungal isolates were subjected to the GTT for presumptive identification of C. albicans, C. stelloidea and C. dubliniensis. A light suspension of the yeast colony was made in 1 ml sterile pooled human sera. The preparation was incubated at 37°C for 3 hours. After incubation, one drop of yeast-serum mixture was put on slide and covered with coverslip. The preparation was observed under the microscope for formation of germ tubes.
iii. **Growth at 42°C:**
All GTT positive isolates were also subjected to incubation at 42°C and presence/absence of growth was noted. *C. albicans* is able to grow at 42°C, while *C. dubliniensis* cannot.

iv. **Morphology on Cornmeal agar (CMA):**
3 to 4 parallel small cuts were made in the CMA plates at 45° angle using a sterile scalpel blade and few colonies of test strain were inoculated. A sterile coverslip was put on the surface of the agar over the cuts. The plates were incubated at 25°C for 72 hours. After incubation, the plate was put on the microscope stage and observed through the coverslip with low and high dry objectives. The characteristic morphology of each isolate was noted. Positive control was used.

v. **Sugar fermentation tests:**
The yeast colony was emulsified in sterile distilled water to obtain turbidity equal to tube number 1 of McFarland standard. 0.2 ml of the suspension was aseptically added to each of the fermentation tubes containing 3 ml of medium. The tube was gently rotated to mix the solutions. All tubes were incubated at 30°C for 7 days. Results were noted in terms of colour change and presence of gas in the inverted Durham's tubes.

vi. **Sugar assimilation pattern:**
Sugar assimilation tests were put up to consolidate the findings of the sugar fermentation reactions. Sugar assimilation was used to differentiate between *C. albicans, C. stelloidea* and other yeasts. A thin yeast suspension was made in 2ml sterile distilled water and the turbidity was matched with tube number 1 of McFarland standard. 0.2 ml of the yeast suspension was added to each tube of previously prepared sugar assimilation medium. Tubes were incubated at 30°C for 7 days, with frequent shaking. Results were interpreted in terms of turbidity (positive for assimilation of that particular sugar) and no turbidity (sugar assimilation negative).
(b). Identification for Mould fungi

The identification of yeast fungi were in accordance to i) characteristic colony morphology on SDA, ii) by seeing the pigment on the back of SDA produced by fungal growth, iii) by making tease-mount preparation and identifying the morphology of conidiophore and fungal head, iv) and in doubtful case, by using micro-slide culture technique and identifying the exact morphology of the hyphae, conidiophore and fungal heads.

i. Colony morphology on SDA:

On SDA, colony of *A. fumigatus* were velvety, with rugosites, and smoky green in colour. The colony of *A. flavus* were velvety at first, then became yellow to green or brown to black in colour.

ii. Pigmentation of the reverse of SDA tubes:

On SDA, the reverse of the culture tube was white to tan for *A. fumigatus*, while the reverse was golden to red brown in colour for *A. flavus*, and without any characteristic change for *A. niger*.

iii. Tease mount Preparation:

One drop of LCB was placed on a clean sterile slide and a small portion of the colony was removed from the culture medium with a help of a L-wire and placed in LCB stain. The mycelia mass of the colony was tease apart gently with the help of dissecting needles. It was then covered with a cover slip, pressed slightly and examined under the microscope.

iv. Micro-slide culture (Dalmau culture plate method):

A sterile microscopic slide was put on a V-shaped bent glass rod positioned on a piece of filter paper in a sterile petri dish (Mackie & McCartney 2007). A 2 mm² small block of SDA was cut using a sterile scalpel blade, scooped out using the tip of a scalpel blade, and placed on the slide. With an L-wire, four quadrant of the agar block were inoculated with the fungal colony to be tested, and a sterile coverslip was put on agar block. The filter paper was moistened with sterile distilled water; the culture dish was covered with its lid and was incubated at 25°C. On appearance of satisfactory fungal growth, the coverslip was removed and placed on a microscopic slide containing few drops of LCB. The agar block was removed, a drop of LCB was placed on the area of growth adhered to the slide, and
a coverslip was placed on it. The other preparation was obtained from the removed coverslip. The two preparations were observed microscopically to see the characteristic morphology of the fungus.

3.6. Antimicrobial Susceptibility Testing.
Antimicrobial susceptibility test was performed for aerobic bacteria by Kirby-Bauer disk diffusion method, for anaerobic bacteria agar diffusion assay were performed and for fungi disk diffusion and broth micro-dilution method were used.

Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method on MHA plates (CLSI 2007).

Antibiotic disks,
Commercially prepared antimicrobial disks purchased from Hi Media Laboratories, Mumbai, India, were used. Antimicrobial disk used were Imepenem(10µg), Aztreonam(30µg), Amoxyclov (30µg), Cefpodoxime(10µg), Cefepime(30µg), Cefoperazone(75µg), Piperacillin/tazobactum(100/10µg), Oxacillin(1µg), Piperacillin(100µg), Cefoperazone/sulbactum(75/10µg), Gentamicin(10µg), Amikacin(30µg), Cefixime(5µg), Ofloxacin(5µg), Cefoxitin(30µg), Ceftazidime(30µg), Ceftazidime/clavulanic acid(30/10µg), Amoxycillin(20µg), Cephotaxime(30µg), Cephotaxime/clavulanic acid(30/10µg), Ceftriaxone(30µg), Chloramphenicol(30µg), Gatifloxacin(5µg), Levofloxacin(5µg), Clindamycin(2µg), Sparfloxacin(5µg), Streptomycin(10µg), Vancomycin(30µg), Tobramycin(10µg), Azithromycin(15µg), Erythromycin(15µg), and Bacitracin (10 units). Staphylococcus species were tested for methicillin resistance by using 1-µg oxacillin disc [NCCLS 2002] and 30-µg cefoxitin disk [Anand et al., 2009]. These discs were stored in sealed cartridges along with desiccant at -20°C in freezer. A small working supply of discs was kept at 4°C for a maximum of one week. On removal from refrigerator for usage, the containers were left at room temperature for about an hour. This will allowed the disc to equilibrate at room temperature thus minimizing the amount of condensation that occurs when warm air comes in contact with cold discs.
Media Preparation.

MHA was autoclaved and 25-30ml was poured into sterile disposable (10 cm in diameter) petri-dishes on a level surface to give a uniform depth of 4mm. The prepared plates were stored in inverted position at 4°C.

Procedure.

Five colonies from overnight growth of test strain were picked up and suspended in a test tube containing sterile nutrient broth. The density was adjusted by comparing the test suspension with 0.5 McFarland turbidity standards as per the recommendation of Clinical Laboratory and Standard Institute (CLSI-2007). Within fifteen minutes on preparation of inoculum, a sterile swab was dipped into the test inoculum and the excess was removed by rotation of swab against the side of tube above the fluid level. The swab was streaked over the surface of medium three times by rotating the plate through an angle of 60° after each application. Then the swab was passed around the edge of agar surface. The inoculated plates were dried at room temperature with the lids closed. Antibiotic disks were applied on the surface of dried inoculated plate using sterile forceps and were gently pressed down to ensure even contact with the medium. Plates were incubated at 37°C for 18 hours.

Interpretation:

Diameter of each zone including the diameter of the disk was measured and recorded in mm with the help of zone scale (HiMedia). The plates were held at 3 inches above black background and the plate was illuminated with reflected light directly from above at 45° angle. End point of inhibition was judged by the naked eye at the point of abrupt diminution of growth or to the point of 80% inhibition as per the recommendation of CLSI-2007. *Staphylococcus* species that were resistant to 1-μg oxacillin disc [NCCLS 2002] and 30-μg cefoxitin disk [Anand et al., 2009] were considered as MRSA positive.

Control strains.

Standard control strains used were:

*S. aureus* (ATCC 25923),

*E. coli* (ATCC 25922) and

*P. aeruginosa* (ATCC 27853).
3.6.2. Susceptibility Testing for Anaerobic bacteria:

Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method on MHA (CLSI 2007).

**Antibiotics used.**

Commercially available antimicrobial powders were purchased from HiMedia Laboratories, Mumbai, India. Antimicrobial powders used were metronidazole, vancomycin, clindamycin, amoxicillin clavulanic acid and imipenem as recommended by CLSI 2007.

**Media Preparation.**

Minimum inhibitory concentrations (MIC) to metronidazole, vancomycin, clindamycin, amoxicillin clavulanic acid and imipenem were obtained by the agar dilution method, performed according to CLSI methods. Antibiotic powders were dissolved in sterile ultra-pure water and added to autoclaved MHA medium to provide increasing 2-fold concentrations of antibiotic and were poured into sterile disposable (10cm in diameter) petridishes on a level surface to give a uniform depth of 4mm.

**Procedure.**

Bacterial suspensions in BHI broth were prepared from fresh overnight cultures (or 40 to 48 hours cultures for slow-growing anaerobic isolates) and were adjusted to a turbidity of 0.5 MacFarland. The 5 µl bacterial suspensions were applied to agar plates (prereduced) using micropipette. Agar dilution plates were incubated in anaerobic jars using anaerobic gas packs (HiMedia, Mumbai, India) and maintained at 35°C for 42 hours to 48 hours.

**Interpretation.**

MIC endpoints were read and susceptibility interpretations were applied using CLSI guidelines.

**Control strains.**

American Type Culture Collection strains of *Bacteroides fragilis* ATCC@ 25285.


**a: For Yeast**

Antimicrobial susceptibility testing was performed by disk diffusion susceptibility testing of yeasts proposed by CLSI document no M44-A.
Antibiotic disks.
Commercially prepared antimicrobial disks purchased from HiMedia Laboratories, Mumbai, India, were used. Antimicrobial disks used were Nystatin (30µg), Amphotericin B (10µg), Fluconazole (25µg) and Itraconazole (80µg).

Media Preparation.
SDA was autoclaved and 25-30ml was poured into sterile disposable (10cm in diameter) petridishes on a level surface to give a uniform depth of 4mm. The prepared plates were stored in inverted position at 4°C.

Procedure.
Five colonies from overnight growth of test strain were picked up and suspended in a test tube containing sterile normal saline. The density was adjusted by comparing the test suspension with 0.5 McFarland turbidity standards. Within fifteen minutes on preparation of inoculum, a sterile swab was dipped into the test inoculum and the excess was removed by rotation of swab against the side of tube above the fluid level. The swab was streaked over the surface of medium three times by rotating the plate through an angle of 60° after each application. Then the swab was passed around the edge of agar surface. The inoculated plates were dried at room temperature with the lids closed. Antifungal disks were applied on the surface of dried inoculated plate using sterile forceps and were gently pressed down to ensure even contact with the medium. Plates were incubated at 37°C for 18 hours.

Interpretation.
Diameter of each zone including the diameter of the disk was measured and recorded in mm with the help of zone scale (HiMedia). The plate was held at 3 inches above black background and the plate was illuminated with reflected light directly from above at 45° angle. End point of inhibition was judged by the naked eye at the point of abrupt diminution of growth or to the point of 80% inhibition as per the recommendation of CLSI document no M44-A.

Quality control.
ATCC 24433 Candida albicans was included each time a set of isolates was tested with each drug.
b: For Mould

Broth micro dilution method was adopted in this study which was proposed by NCCLS 2002, based on document no M-38A.

**Antibiotic.**

Commercially available antimicrobial powders (nystatin, amphotericin B, fluconazole & Itraconazole) were purchased from HiMedia Laboratories, Mumbai, India.

**Media Preparation.**

The medium used for the sensitivity of moulds were Roswell Park Memorial Institute 1640 (RPMI-1640) medium with 0.165 mol/L of 3-(N-morpholino) propanesulfonic acid (MOPS) purchased from HiMedia, Mumbai, India. The powdered RPMI-1640 (10.4g) was dissolved in 900ml of sterile water. MOPS was added to a final concentration of 0.165 mol/L and stirred until dissolved. The pH was maintained at 7 using 1mol/L sodium hydroxide. Additional water was added to make final volume of 1 liter. It was filter sterilized and stored at 4°C until use. The highest desired conc was 1600 μg/ml. Hence 4.8 mg of antifungal agent was weighted and dissolved in 3ml of Dimethyl Sulfoxide (DMSO) (assuming 100% potency of drug). The dilution series were in the range of 16 μg/ml to 0.0313 μg/ml for all the drugs tested.

**Procedure.**

Five colonies from overnight growth of test strain were picked up and suspended in a test tube containing sterile normal saline. The density was adjusted by comparing the test suspension with 0.5 McFarland turbidity standards. Within fifteen minutes on preparation of inoculum, a sterile swab was dipped into the test inoculum and the excess was removed by rotation of swab against the side of tube above the fluid level. The swab was streaked over the surface of medium three times by rotating the plate through an angle of 60° after each application. Then the swab was passed around the edge of agar surface. The inoculated plates were dried at room temperature with the lids closed. Antifungal disks were applied on the surface of dried inoculated plate using sterile forceps and were gently pressed down to ensure even contact with the medium. Plates were incubated at 37°C for 18 hours.

**Inoculum preparation.**

Conidia formation was induced by growing the isolates on CMA at 35oC. Fungal colonies were covered with 1ml normal saline and the conidia were harvested by probing the
colonies with the tip of a sterile pasture pipette. The resulting mixture of conidia and hyphae was transferred to a sterile tube. Heavy particles of the suspension (if present) were allowed to settle for 3.5 min, and the upper homogenous suspension after vortexing for 15 seconds was adjusting spectrophotometrically to optical densities that ranged from 0.09 to 0.11 at 625 nm. The inoculums suspension was diluted 1:50 in RPMI, which corresponds to two times the density (2X) needed for the test (approximately 4x10⁴ to 5x10⁴ CFU/ml). The test inoculums were made in corresponding diluted inoculums suspension. A growth control well containing RPMI-1640 growth without an antifungal agent was put each time a test was performed. The drug concentration (2x) of Nystatin, Amphotericin B, Fluconazole and Itraconazole were dispensed into well 1 to 10 of microtiter plate in 100 µl volume in decreasing order. Each well of plate inoculated with 100 µl of corresponding (2x) suspension of diluted inoculum. The wells serve as controls contain 100 µl of drug free medium and are inoculated with 100µl of corresponding suspension of inoculum (2x). An additional row was also used to perform sterility test. The plate was incubated at 35°C for 48h.

**Interpretation.**

The growth of each well was compared with that of the growth control with a reading mirror. The results were scored as “0” for optically clear, “1” for slightly hazy, “2” for prominent decrease in turbidity, “3” slightly reduction in turbidity and “4” as no reduction of turbidity.

**Quality control.**

Two QC organisms v.i.z A flavus, ATCC 204303 and A fumigatus ATCC 204305 were included each time a set of isolates was tested with each drugs. Sterility control was also performed by adding 1 ml un-inoculated drug free medium.

**3.7. ESBL Detection**

**3.7.1. Phenotypic ESBL detection.**

The phenotypic detection of ESBLs in the clinical isolates involves screening and confirmatory tests as recommended by CLSI 2007.

**3.7.1.1. Phenotypic screening methods for ESBL producer.**

All the Gram’s negative bacteria isolated from DFU were first screened for ESBL
production by using extended spectrum β- lactam antibiotics disk diffusion (Paterson and Bonomo, 2005). Cefpodoxime, ceftazidime, aztreonam, cefotaxime, and ceftriaxone were tested as per CLSI guidelines for the disk diffusion method for ESBL detection. The use of more than one of these agents for screening improves the sensitivity of detection. The zone diameters indicate suspicion for ESBL production.

**Procedure.**

Inoculum with turbidity equivalent to 0.5 McFarland standards was prepared from colonies on agar plates. MHA plates were inoculated by swabbing them with a sterile cotton swab. With a sterile forceps ceftazidime, cefotaxime ceftriaxone and aztreonam disks were placed on the MHA plate and plate was incubated at 35°C for 18-24 hours. The quality control strain *K. pneumoniae* ATCC 700603 (ESBL producer) was used.

**Interpretation of results.**

Zones of inhibition (in mm) against the respective antibiotic indicate potential ESBL production. If any strain was suspected to be an ESBL producer then phenotypic confirmatory test was done.

- Ceftazidime ≤22mm
- Aztreonam ≤27mm
- Ceftriaxone ≤25mm
- Cefotaxime ≤27mm

### 3.7.1.2. Phenotypic confirmatory method for ESBL production

Cephalosporin/Clavulanate Combination Disk (Paterson and Bonomo, 2005). The CLSI recommends that the disk tests be performed with confluent growth on Mueller- Hinton agar. A difference of 5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disk is taken to be phenotypic confirmation of ESBL production. The tazobactum with piperacillin disc method was adopted (Shahid et al., 2007). Antibiotic discs used were

- Ceftazidime (30 µg)
- Ceftazidime + clavulanic acid (30/10 µg)
- Cefotaxime (30 µg)
- Cefotaxime + clavulanic acid (30/10 µg)
- Piperacillin (100 µg)
- Piperacillin + tazobactum (100/10 µg)
- Cefoperazone (75 µg)
- Cefoperazone + salbactam (75/10 µg)
Procedure:
MHA plates were used for the phenotypic confirmation. Plates of test and control organisms were inoculated as mentioned in disk diffusion method. Pair of disks containing ceftazidime / clavulanic acid, piperacillin / tazobactam, and cefoperazone/ salbactam were placed on the plate inoculated with the test organism. Inoculum, incubation conditions and incubation time were same as in standard disk diffusion method. The quality control strain K. pneumoniae ATCC 700603 (ESBL producer) was used.

Interpretation of result.
An increase in the zone diameter of ≥ 5mm for either antimicrobial agent tested in combination with clavulanic acid / salbactam / tazobactam / in comparison to its zone when tested alone was taken as indicative of ESBL production.

3.7.3. Genotypic methods of ESBL genes detection.
Genotypic identification of ESBL was done using polymerase chain reaction for bla_{CTX-M}, bla_{TEM} and bla_{SHV} genes. The genotypic methods involve i) plasmid isolation, ii) PCR amplification of bla gene, iii) preparation of reaction mixture, iv) cycling condition for bla gene, v) preparation of TAE buffer and vi) Agarose gel electrophoresis which were adopted from Shahid et al., (2009).

3.7.3.1. Plasmid isolation by Boiling and Chilling:
In this method, about 0.5-1.0 ml of BHI broth culture was subjected to vigorous heating in a boiling water bath for 10 minutes and then snap chilled. From this about 5 µl was used as a template in PCR.

3.7.3.2. PCR amplification of bla gene:
The primers used in this study were adopted from the study of Shahid et al., [2009] and it was obtained from Operon Biotechnologies Nattermannallee 1, 50829 Cologne Germany. The details of the primer used in this study, their sequence (5′ to 3′) with size of amplified product was as follows:-
Materials and Methods

<table>
<thead>
<tr>
<th>Targets</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Amplified product size</th>
</tr>
</thead>
</table>
| *bla*<sub>CTX-M</sub> | F-ATGTGGAGYACCACTAAARGT  
R-TGGGTRAARTARGTSACCAGA | 596 bp |
| *bla*<sub>TEM</sub>  | F- KACAATAACCCTCRTAAATCC  
R-AGTATATATGAGTAAAACCTTG | 936 bp |
| *bla*<sub>SHV</sub>  | F- TTTATGCGGYTCATCAACG  
R-GCTGCGGGCCGGATAACG | 963 bp |

Y Wobble (C + T); R Wobble (A + G); S Wobble (C + G); K Wobble (G + T)

3.7.3.3 Reaction mixture and Cycling Conditions:
The PCR tube containing the reaction mixture was flash spun in a micro centrifuge to get the reactants at the bottom. The reactions were performed in a master cycler Gradient thermocycler (BioRad, Germany) with a preheated lid.

A). Detection of *bla*<sub>CTX-M</sub>:
For the detection of *bla*<sub>CTX-M</sub>, the reaction mixture and PCR cycling condition were as follows:

**Preparation of Reaction Mixture.**
The PCR reaction mixture was prepared using the following reagents in the desired amount:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity, for 50 µl of reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer Forward</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Primer Reverse</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>2X PCR Master Mix</td>
<td>25 µl</td>
</tr>
<tr>
<td>Template DNA solution</td>
<td>5 µl</td>
</tr>
<tr>
<td>Water, nuclease free</td>
<td>19.8 µl</td>
</tr>
</tbody>
</table>

**Cycling Conditions for *bla*<sub>CTX-M</sub>**:  
PCR was carried out in a thermal Cycler with a first cycle of denaturation at 94°C for 7 min, then 35 cycles of denaturation at 94°C for 50 sec, annealing at 50°C for 40 sec and elongation at 72°C for 60 sec. The PCR was completed by a final elongation step at 72°C for 5 min.
B). Detection of bla$_{SHV}$.

For the detection of bla$_{SHV}$, the reaction mixture and PCR cycling condition were as follows:

**Preparation of Reaction Mixture:**

The PCR reaction mixture was prepared using the following reagents in the desired amount:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity, for 50 µl of reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer Forward</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Primer Reverse</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>2X PCR Master Mix</td>
<td>25 µl</td>
</tr>
<tr>
<td>Template DNA solution</td>
<td>5 µl</td>
</tr>
<tr>
<td>Water, nuclease free</td>
<td>19.8 µl</td>
</tr>
</tbody>
</table>

**Cycling Conditions for bla$_{SHV}$:**

PCR was carried out in a thermal Cycler with a first cycle of denaturation at 95°C for 15 min, then 34 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 2 min and elongation at 74°C for 3 mins. The PCR was completed by a final elongation step at 72°C for 10 min. After the reaction, tubes with PCR products were held at 4°C until further analysis / confirmation by Agarose gel electrophoresis (2% agarose).

C). Detection of bla$_{TEM}$

For the detection of bla$_{TEM}$, the reaction mixture and PCR cycling condition were as follows:

**Preparation of reaction Mixture:**

The PCR reaction mixture was prepared using the following reagents in the desired amount:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity, for 50 µl of reaction mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer Forward</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>Primer Reverse</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>2X PCR Master Mix</td>
<td>25 µl</td>
</tr>
<tr>
<td>Template DNA solution</td>
<td>5 µl</td>
</tr>
<tr>
<td>Water, nuclease free</td>
<td>19.8 µl</td>
</tr>
</tbody>
</table>
Cycling Conditions for blaTEM:
PCR was carried out in a thermal Cycler with a first cycle of denaturation at 95°C for 15 mins, then 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 2 mins and elongation at 72°C for 3 mins. The PCR was completed by a final elongation step at 72°C for 10 mins.

3.7.3.4. Preparation of TAE buffer
The TAE buffer was prepared using the following chemicals

<table>
<thead>
<tr>
<th>Reagents required</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>242 gms</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1 ml</td>
</tr>
<tr>
<td>0.5M Disodium EDTA (pH – 8.0)</td>
<td>100 ml</td>
</tr>
<tr>
<td>Double distilled water to make</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

3.7.3.5. Agarose gel electrophoresis:
Principle:
Gel electrophoresis is a procedure for separating a mixture of molecules through a stationary material (gel) in an electrical field. The gel is jello-like material, usually agarose, a substance derived from seaweed. Wells were prepared in the gel. DNA solutions (mixtures of different sizes of DNA fragments) are loaded in each well in the gel. The gel matrix acts as a sieve for DNA molecules. Small molecules move more easily through the holes than large molecules. As the separation process continues the separation between the larger and smaller fragments increases. Molecular weight markers were electrophoresed with sample DNA.

Preparation of Agarose gel:
Agarose gel was prepared by boiling molecular biology grade agarose (Sigma Aldrich, India) in 0.5X TAE buffer to dissolve it completely. After cooling it to about 50°C, Ethidium bromide (Sigma Research Laboratory, India) was added to the agarose solution to a final concentration of 0.5 µg /ml. Before dissolving the agarose, the gel-casting platform was placed on a levelled surface with the help of gel caster. The gel comb was then placed across the gel-casting platform, so that the teeth of the comb
remained 1mm above the base of the platform. The molten agarose was then poured on to the gel-casting platform and it was kept undisturbed for about an hour to solidify the gel. After the gel solidified the comb was taken out and the tray was removed from caster. The set gel with the gel-casting platform was then submerged in the electrophoresis tank (BioRad, India) with the wells at the cathode end of the tank with sufficient quantity (about 1 mm level) of electrophoresis buffer (TAE, 0.5x) above the surface of the gel.

PCR product (10 µl) was mixed with 6X bromo-phenol blue loading dye (2 µl) and was loaded into the respective wells. First and last well was used as marker ladder (100bp) purchased from Fermentas. Electrophoresis was performed at 6 V/cm and the progress of mobility was monitored by the migration of the dye. At the end of electrophoresis the gel was visualized under Gel documentation system (BioRad, Germany) for the bands of desired molecular weight.

The baseline data was analyzed using SPSS version 17.0 for descriptive statistics. Quantitative variables were expressed as mean±sd while qualitative variables were expressed as percentage (%). Continuous variables were compared using 2 sample t tests for independent samples. Odds ratios and 95% confidence interval (CI) were reported for independent variables associated with the outcome variable. P-values less that 0.05 were considered significant. The differences between the groups were calculated with Student t or the nonparametric U-Mann-Whitney tests & Shapiro-Wilk test was used to evaluate normality of variables. A pearson correlation analysis & multiple stepwise linear regression was used to assess the association between all variables and grading of diabetic foot ulcer that independently predicted the risk of foot ulcer development with a P < 0.05. For the CCRe study, independent sample t-test for equality of variance was used to compare variables of patients. Differences in proportions were compared using chi-square test, Holm-Sidak method and Kruskal-Wallis method. Analyses were conducted on Sigma plot 11.0 software. Values were given as mean±standard error (SE) and/or n (%) otherwise indicated. p values less than 0.05 were considered statistically significant.
To study of risk factors for amputation, patients were divided into two groups, Group A: amputation group (patients underwent major and minor amputations) and Group B: non-amputation group. Quantitative variables were expressed as mean ± sd. The normality of the distribution of each continuous variable was assessed using the Kolmogorov-Smirnov test. If normality was established, Odds ratios (strength of association) and Risk ratio (the probability of the amputation) with 95% confidence interval (CI) were reported for independent variables associated with the outcome variable: amputation and non-amputation. All analysis was performed using SPSS 17.0 software. P-values less than 0.05 were considered significant.

Cefotaxime resistant patients were divided into a two groups (bla gene positive and bla gene negative). Quantitative variables were expressed as mean±sd. Odds ratios (strength of association) and Risk ratio (the probability of association) with 95% confidence interval (CI) were reported for independent variables associated with the outcome variable: Bla gene positive and negative. All analysis was performed using SPSS 17.0 software. P-values less than 0.05 were considered significant.