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Isolation, molecular characterization and antibiogram of bacteria isolated from diabetic foot ulcers

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Diabetic foot ulcers; *Staphylococcus aureus;* Multiple antibiotic resistance; Phylogenetic tree.

A B S T R A C T

Diabetic patients have increased risk of foot ulcers leading to minor or major amputation. This is habitually caused either by neuropathy or ischaemia, in few cases by both. Initial cause for such ulcers may be due to acute mechanical or thermal trauma or by repeated mechanical stress. Multidrug resistant aerobes have been closely related to diabetic foot infections. A high percentage of *Staphylococcus aureus* isolates were reported in hospitals associated with ulcer infections (Sanjay *et al*., 2013). In this study, swab samples from diabetic patients' foot ulcers from hospitals in and around Chennai were collected. Out of 22 isolates, 4 strains showed high Multiple Antibiotic Resistant (MAR) index. 16S rRNA gene was amplified using PCR technique in all the 4 strains and they were characterized as *Staphylococcus aureus*, *Morganella morganii*, *Acinetobacter baumannii* and *Acinetobacter* Spp. Phylogenetic tree for each of the isolate was constructed to analyze its evolutionary relationship with closely related species.

Introduction

According to American Diabetic Association, Diabetes mellitus (DM) is a disease caused by metabolic disorder (Sociedade Brasileira de Diabetes, 2012). It is classified in to two basic forms Type I and Type II diabetes. Type I DM is caused by immunological deficiency in pancreas leading to insufficient insulin production (World Health Organization, 1999). Type II DM occurs when the body becomes insulin resistant or it does not respond to the insulin produced (Uma Makheswari and Sudarsanam, 2012). Foot lesions and foot ulcers are the most commonly observed impediments with diabetic patients. Pervasiveness of these ulcers in diabetic

patients is as high as 25% and requires constant monitoring and patients have to visit a hospital frequently which is exorbitant (Albert *et al*., 2012). The concurrence of neuropathy, ischemia and immune function disorder in diabetic patients favors rigorous infection in the lower limbs (Vasnessa *et al*., 2006). This rigorous infection spreads by softening of the tissue and bones leading to amputation of the lower limbs. Making early diagnosis and adequate treatment is necessary although diagnosing foot infection at an early stage in a diabetic patient is strenuous (Albert *et al*., 2012).

Methicillin resistant *Staphylococcus aureus* (MRSA) remains the predominant pathogen in diabetic foot ulcers (Sanjay *et al*., 2013). Other aerobes associated with diabetic foot ulcer involve *Staphylococcus epidermidis, Streptococcus* Spp., *Pseudomonas aeruginosa, Enterococcus* Spp., *Coliform bacteria* and *Acinetobacter baumannii* (Anne *et al*., 2001; Mendes *et al*., 2012). Clinical trials on a double blind bases have shown that MRSA and its associates are sensitive to Sparfloxacin, Levofloxacin, Ofloxacin, Ciprofioxacin, Amoxicillin-clavulanic acid, Cephalexin, Cefuroxime, Doxycycline and Clindamycin (Ellie *et al*., 1996). Antibiotic resistance has been classified by the World Health Organization as one of the three major public health threats of the 21st century (Lévesque *et al*., 1995). Therefore, antibiotic options in the treatment of diabetic foot ulcers against these organisms are extremely limited.

The most common genetic marker, the 16S ribosomal RNA gene is present almost in all bacteria and its functions have not changed over a long period of time (Michael and Sharon, 2007; Olsen and Woese, 1993). Phylogentic tree reveals its evolutionary relationship among organisms. PCR based molecular detection is an ideal technique due to its accurateness and rapid detection which is irrevocable for diagnosing antibiotic resistance. Hence, this study was undertaken to isolate antibiotic resistant microorganisms from infected diabetic foot and to find out the occurrence of 16S ribosomal RNA and to predict its evolutionary relationship.

Materials and Methods

Sample collection

Swab samples were collected from diabetic patients' infected foot ulcers in and around Chennai. All samples were collected from hospitals in a sterile container and transported to the laboratory within two hours from collection and stored at 4˚C, until processing of the samples. The collected swabs were directly inoculated into nutrient broth and incubated overnight at 37˚C. The isolates present in the swab samples were allowed to grow, from which drug resistant micro organisms were analyzed.

Isolation of the microbes

To screen and isolate the different multi resistant micro organisms from the enriched culture, two different media were prepared using the following composition:

Sheep blood agar

Pancreatic digest of casein-15.0 g, Papaic digest of soybean meal-5.0 g, Sodium chloride-5.0 g, Agar-20.0 g, Yeast extract-5.0 g, Hemin-0.005 g, Vitamin K10.01 g, L-Cystine-0.4 g, Sheep blood, defibrinated-5% and distilled water-1000 ml. Loop full of cultures were then streaked on to Sheep blood agar plates and incubated overnight

for the enumeration and single colony isolation.

Mannitol salt agar

Pancreatic digest of casein-5 g, Pancreatic digest of animal tissue–5 g, Beef extract–1 g, D-Mannitol-10 g, Sodium chloride–75 g, Phenol red–25 mg, agar-15% and distilled water–1000 ml (pH 7.4 was maintained). Loop full of cultures were then streaked on to mannitol salt agar plates and incubated overnight for the enumeration and single colony isolation. The isolates were preliminarily grouped according to colony morphology and subjected to biochemical tests.

Microbial and biochemical tests

The isolates from both sheep blood plates and mannitol plates were subjected to biochemical characterization. Microbial and biochemical parameters, Gram staining, catalase, oxidase and motility tests were performed. Phenotypic characterization tests, indole test, methyl red test, Voges-proskaur test, citrate utilization test, nitrate reduction test and urease tests were also performed.

Antibiotic test screening (Kirby Bauer's method)

In order to determine the anti-biograms of the isolates, pure cultures were inoculatedin 4–5 ml of nutrient broth and incubated at 37ºC for 24 hrs. The diluted inoculums were swabbed on the surface of Muller Hinton agar (Hi-Media Pvt. Ltd. India) plate by swab method. Antibiotic discs were placed on the surface and allowed to stay at 37°C in the incubator and their antibiotic resistance was assessed. All the 22 strains were subjected to seven commonly used antibiotics and their percentage of resistance was calculated.

Multiple antibiotic resistances (MAR) index (Krumperman, 1985) was calculated by the formula (Hinton *et al*., 1985).

MAR index = y/nx.

Where, y = total number of resistance scored; n = number of isolates; x = total number of antibiotics tested.

Screening of 16S rRNA gene

The genomic DNA was extracted from the isolated strains using standard phenol: chloroform method (Sambrook *et al*., 1989). 16S rRNA sequence was amplified using universal primers by PCR method. Amplification was carried out in a 20 µl reaction set up containing 0.3 µM of each primer, 0.2 mM deoxynucleotide triphosphates, 100 ng of template DNA sample and 1U of Prime TaqDNA polymerase (Genetbio, Korea). The reaction tubes were subjected for Thermal cycling reactions consisting of an initial denaturation (5 min at 94°C) followed by 32 cycles of denaturation (1 min at 94°C), annealing (45 s at 48°C), and extension (1 min at 72°C), with a final extension (10 min at 72°C). The PCR product was purified (QIAquick PCR purification kit, Qiagen, Madrid, Spain) and analyzed by DNA sequencing (3730 DNA sequencing analyzer, ABI). The nucleotide sequences obtained were compared to the 16S rRNA of *Staphylococcus aureus*, *Morganella morganii*, *Acinetobacter baumannii* and *Acinetobacter* Spp. were compared with wild type sequence CP003808, CP004345, CP000863 and CP000521, respectively using BLAST tool. These were given as an input in MEGA5 software and phylogenetic tree was constructed.

Result and Discussion

Swab samples were collected from diabetic patients' infected foot. Isolation and biochemical studies resulted in the characterization of the isolates. The biochemical test in Tables 1 and 2 indicates the 22 isolated strains. Out of these results, nine cultures were *Staphylococcus aureus* indicating its prominences in the patients infected foot. Multiple antibiotic resistances were calculated for all the 22 strains and its percentage was established. A graph illustrating the MAR index of the isolates is shown in Graph 1. A maximum resistance of 78.5% was seen in strain 5 and a minimum of 14.2 was seen for strain 6, 7 and 13. 100% sensitivity was observed in 5 strains.

Out of the 22 isolates, only four isolates showed soaring antibiotic resistance. Only those cultures were taken for characterization. 16S rRNA gene specific PCR amplification was done and bands were visualized at 1500bp. All the four samples were sequenced and the isolates were confirmed as *Staphylococcus aureus* (strain 5), *Morganella morganii* (Strain 17), *Acinetobacter baumannii* (Strain 19) and *Acinetobacter* Spp. (Strain 20). All obtained sequences were submitted to NCBI genebank and their accession numbers are KC425220, KC188661, KC188662 and KC193593 respectively.

FASTA format files containing the sequences and its closely related species were taken as reference and aligned. These sequences were then used to construct evolutionary tree. A phylogenetic tree was constructed from these data using MEGA version 5 software (Koichiro *et al*., 2011). Maximum likelihood tree was constructed for all the four isolates respectively as shown in Figures 1–4.

Antibiotic resistance in bacteria is a major increasing issue in diabetic foot infections. A number of risk factors related to antibiotic resistance have been shown in varies studies. Such studies include presence of osteomylelitis, antibiotic dosage, its duration and number of hospitalization for the same ulcer (Renato *et al*., 2012).

The main purpose of this study was to isolate antibiotic resistant microbes from diabetic patients' foot ulcers and uncover its evolutionary relationship among its closely allied species. Initially swab samples from diabetic patients' foot ulcers were collected in and around Chennai. Gram negative *Staphylococcus aureus* is a predominant pathogen isolated among the other organisms from diabetic foot infections (Aragon *et al*., 2008; Shao *et al*., 2010). Almost 50% of *Staphylococcus aureus* isolates are methicillin-resistant *S. aureus* (MRSA). Other frequently occurring micro organisms are *Pseudomonas aeruginosa, Morganella morgani, Klebsiella pneumonia, Escherichia coli, Providencia rettigeri, Proteus vulgaris, Klebsiella oxytoca, Citrobacter diversus, Acinetobacter baumanii, Streptococcus agalactiae* and *Enterobacter*, although their percentage of incidence is slightly lesser than *Staphylococcus aureus* (Vasnessa *et al*., 2006).

In the present study, 22 cultures were enumerated in Sheep blood plates and Mannitol salt agar plates. All cultures were screened for their biochemical characterization as shown in Table 1. Eight cultures showed individual cocci resembling the morphology of Staphylococci and the rest showed prevalence of six cultures of *Morganella*, four cultures of *Enterobacter* and four

Figure.1 Maximum likelihood tree for *Staphylococcus aureus*

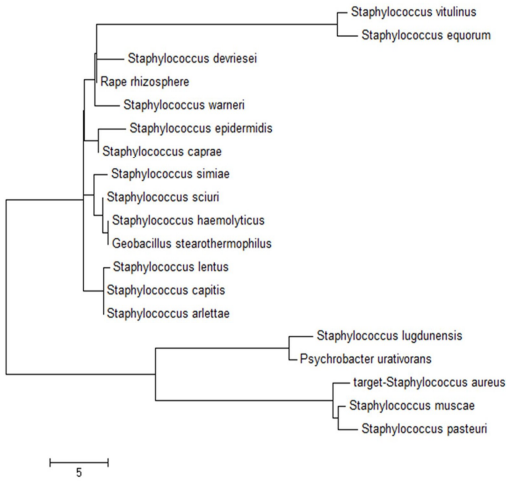


Figure.2 Maximum likelihood tree for *Morganella morganii*

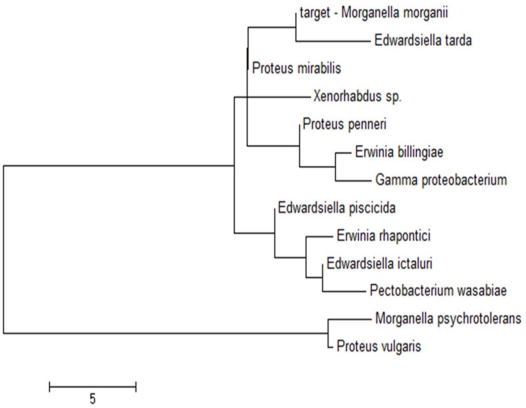


Figure.3 Maximum likelihood tree for *Acinetobacter baumannii*

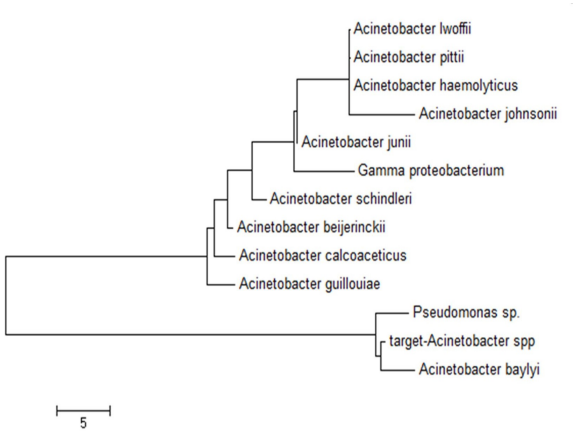
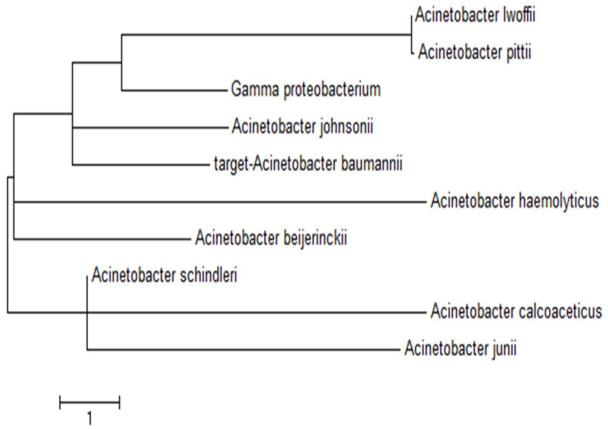


Figure.4 Maximum likelihood tree for *Acinetobacter* Spp**.**



Graph.1 Multiple antibiotic resistance for all the 22 isolates against 7 antibiotics

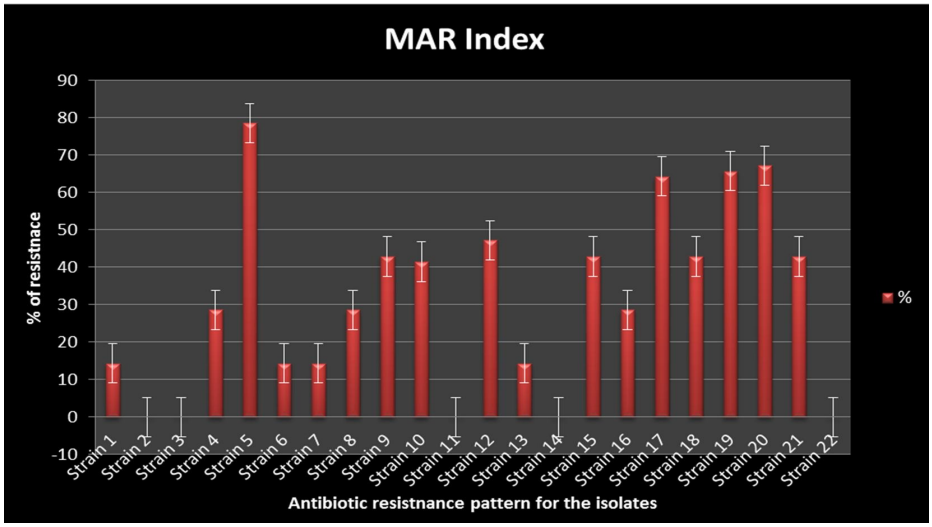


Table.1 Biochemical tests results for isolates 1 to 11 are shown. Strain is mentioned as "S".

Test	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11
Gram staining	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Urease	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
Oxidase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
Indole	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
Methyl red	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
Voges-Proskauer reaction	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Citrate	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve
Nitrate reduction	+ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Mannitol	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Sucrose	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve

Table.2 Biochemical tests results for isolates 12 to 22 are shown. Strain is mentioned as "S"

Test	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22
Gram staining	-ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	-ve
Catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Urease	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve
Oxidase	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve
Indole	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
Methyl red	-ve	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
Voges-Proskauer reaction	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve
Citrate	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	-ve
Nitrate reduction	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	+ve	+ve
Glucose	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Mannitol	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve
Sucrose	+ve	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	+ve	-ve

cultures of *Acinetobacter*. This was in accord with Caputo *et al.*, (1994), who noted that mild diabetic foot infections are often caused by *Staphylococci* and *Streptococci*.

The isolates obtained were subjected to antibiotic resistance analysis using antibiotic discs and were categorized based upon the diameter of the zone. MAR index was calculated for all the strains as shown in Graph 1. Strain 5, Strain 17, Strain 19 and Strain 20 were observed with elevating percentage of resistance 78.5%, 65%, 67.2% and 68.3% respectively, while the rest were below 50%.5 stains on the other hand showed poor antibiotic resistance.

Amplification of 16S rRNA is a gold standard to explore bacterial phylogeny and taxonomy with more accurate measure of time (Michael and Sharon, 2007). Characterization of the resistant cultures confirms the strain 5, 17, 19 and 20 as *Staphylococcus aureus*, *Morganella morganii*, *Acinetobacter baumannii* and *Acinetobacter* Spp., respectively. The present study corroborates to previous studies in which Gram-positive aerobes were predominant in diabetic foot infections (Dang *et al*., 2003; Mantey *et al*., 2000). Interestingly *Morganella morganii* was first identified as a cause for foot ulcers only in 2006 (Deutsch *et al*., 2006) and found only in 10% of foot ulcers (Vasnessa *et al*., 2006) were found to be predominant a butting to *Staphalococcus* in our study.

Acinetobacter baumanni is known for nosocomial or hospital acquired infection and is rarely found in human skin infections. In our study, two resistant cultures were found to be as *Acinetobacter* Spp. Possibly, its potential to cause infection is due to its various virulence factors that helps in its survival in hospital environment and especially in diabetic patients (Bergogne-Berezin and Towner (1996).

With all the sequences obtained, individual phylogenetic tree was constructed for each of the resistant isolates. Figure 1 illustrates the maximum likelihood tree for *Staphylococcus aureus*. It shows that the target-*Staphylococcus aureus* originates from a single ancestral root. However, it is branched with *S. lugdunesis* and forms monophyletic sister clade with *S. muscae* and *S. pasteuri.* In addition, *S. aureus* is closely related to *P. Urativorans*, a genus within gamma-proteobacteria which is an opportunistic microorganism infecting human tissue (Bozal *et al*., 2003). This finding depicts the underlined relation between pathogens causing the same effect on diabetic ulcers.

Figure 2 illustrates phylogenetic tree for *Morganella morganii*, which was closely clustered to *Edwardsiella tarda*. It forms sister group with *P. penneri*, *Erwinia billingiae* and *Edwardsiella piscicida* corroborating previous studies of Kauffmann (1954) who first revealed that *M. morganii* is closely related to *Proteus* and *Providencia*. In addition, it is a surprise that *Edwardsiella tarda* and *Edwardsiella piscicida* originally causing body ulcers and hemorrhagic septicaemia in marine organisms (Putanae *et al*., 2003) are found closely related to *M. morganii* which causes ulcers in Homo-sapiens.

Figures 3 and 4 exemplify the evolutionary tree of *Acinetobacter baumannii* and *Acinetobacter* Spp. They clustered with their own sub-species showing no high evolutionary traits acquired except for showing a branch with *Pseudomonas* Spp. and *Gamma proteobacterium*.

In this study, antibiotic resistant strains were isolated from diabetic patients' foot ulcers and 16S rRNA gene was amplified to characterize the isolates. Furthermore, phylogenetic analysis was done which is necessary to identify the immediate sister species which causes severe skin infections in diabetic patients leading to amputation. It implies that early diagnosis and treatment options can be done if prior knowledge of bacterial prevalence is understood.

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