Biofilm Forming Abilities of Microorganisms Associated with Diabetic Wound Infection: A Study from a Tertiary Care Hospital

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Diabetes mellitus, a chronic metabolic disease is increasing worldwide. Diabetic foot infections are one of the most feared and bothersome complications of diabetes caused by different genera of bacteria. There is an increasing evidence which demonstrates the presence of biofilm formers in chronic diabetic foot ulcers which contribute to the development of antibiotic-resistant strains and treatment failure. The present study aimed at isolating bacteria from diabetic wounds, to check for its antibiotic susceptibility and biofilm forming ability. From the diabetic wounds, isolates belonging to the genera of Staphylococcus, Pseudomonas, Klebsiella, Escherichia, Vibrio, Acinetobacter and Citrobacter were recovered. To the best of our knowledge, Vibrio parahaemolyticus was isolated for the first time from diabetic ulcer. Antibiotic sensitivity profile of the organism infers the presence of multidrug-resistant strains. Majority of the bacteria isolated were found to be biofilm formers. High biofilm formers were observed in strains of P. aeruginosa, S. aureus and Klebsiella spp. There was a significant association between incubation time and intensity of biofilm formation in P. aeruginosa \( \chi^2 \) \( (p<0.05) = 0.001 \), Staphylococcus spp. \( \chi^2 \) \( (p<0.05) = 0.023 \) and Acinetobacter spp. \( \chi^2 \) \( (p<0.05) = 0.018 \). The presence of biofilm forming multidrug-resistant bacteria infers the chronic nature of diabetic wounds.

Keywords: Diabetic wound, Poly microbial infection, multidrug-resistant bacteria, Biofilm.
infection have acquired resistance to the available antibiotics making treatment regimen complicated.

The polymicrobial community that cause infection can produce extracellular polymeric substances called biofilms. Biofilm formed performs a dual function in acting as a physical barrier for biological and antimicrobial substances and also facilitate adhesion to surfaces. In recent years, biofilms have gained as an important means of survival of microorganisms in hostile environment. Bacteria in biofilm exchange genetic material, communicate with each other, which often result in altered phenotype of bacteria which influences the wound healing process. Chronic diabetic foot infection due to biofilm formers contribute to the development of antibiotic resistant strains and treatment failure. Though there are many studies worldwide on this topic, hardly few studies have been conducted in Mangaluru region focusing on the biofilm forming abilities of the organisms isolated from foot wounds. Against this background, our study focused on isolating bacteria from diabetic wound infection, checking for their antibiotic susceptibility and also biofilm forming abilities of these pathogen.

**MATERIALS AND METHODS**

**Sample collection**

Clinical samples (wound swab) from patients with a history of diabetes was collected from Justice K.S Hegde hospital, Deralakatte, Mangaluru, by taking clearance from the institutional ethics committee (INST.EC/2017-18/003) before the commencement of this work. Patient consent was taken before sample collection and was anonymized. Sample collection was carried for a period of 3 months between January and March, 2018. Collected swabs were enriched in brain heart infusion broth for the period of 8 hours.

**Selective Isolation**

Culture from the enrichment media was streaked onto nutrient agar plates and colonies that developed were inoculated onto different selective media viz., cetrimide agar, mannitol salt agar, MacConkey agar, Leeds *Acinetobacter* agar and thiosulphate citrate bile salt sucrose agar.

**Identification of bacteria**

Phenotypic identification was done by performing Gram staining and an array of biochemical tests. Genotypic identification was carried out by polymerase chain reaction using genus and species specific primers. The list of primers used in the study is given in Table 1.

**Antibiotic susceptibility testing**

All the confirmed isolates were subjected to antibiotic susceptibility test by employing Kirby Bauer disc diffusion method. Antibiotics norfloxacin, imipenem, tetracycline, gentamicin, amoxycilav, ampicillin, amoxicillin, ciprofloxacin, cefoxitin, ceftaxime were used for Gram negative bacteria, vancomycin, penicillin G, amoxycilav, azithromycin, tetracycline, trimethoprim, and oxacillin were used for Gram positive bacteria and cefoperazone, piperacillin, levofloxacin, gentamicin, amikacin, imipenem, aztreonam, cefoperazone/sulbactam, piperacillin/tazobactam, ceftazidime, netillin, ciprofloxacin, tobramycin were used for *P. aeruginosa*. The zones of inhibition (mm) that developed after an incubation period of 24 h were measured.

**Qualitative and quantitative assay for biofilm**

**Congo red method**

Qualitative detection of biofilm formation was carried out by Congo red method. Biofilm formers formed black colonies with a dry crystalline consistency.

**Microtitre plate method**

Biofilm quantification was carried out according to the method of O’Toole and Kolter with slight modification. In a microtitre plate, 100 µl of the diluted culture was taken and incubated for 24 h at 37ºC. Using PBS of pH 7.4, the adherent cells were washed thrice. 125 µl of 0.1% freshly prepared crystal violet was added to the dried pellet, and incubated for 10 min. 200 µl of 30% acetic acid was added to the stained and washed pellet, and incubated for 15 min for stain solubilisation. To a fresh plate, 100 µl from the well was transferred and optical density was measured at 600 nm in an ELISA reader (Biorad, USA). Reduction in the biofilm formation was measured in terms of percentage inhibition as [(OD of control – OD of treated)/ OD of control] x100. The biofilm formed by the confirmed isolates was compared with standard culture of different organisms. The biofilm formers were grouped as weak biofilm formers (OD600 0.071 – 0.142), moderate biofilm formers (OD600 0.142 – 0.284) and high biofilm formers (OD600 ≥ 0.284).
Biofilm formation was quantified at different time intervals (24, 48 and 72 h).

RESULTS

Isolation and identification of bacteria

Out of 133 colonies found growing on the selective media, 36 developed on Mac Conkey agar, 26 on thiosulphate citrate bile salt sucrose agar, 27 on cetrimide agar, 36 on mannitol salt agar and 8 on Acinetobacter agar. The development of bacteria was predominantly more on mannitol salt agar and cetrimide agar indicating the presence of large number of Staphylococcus spp. and P. aeruginosa respectively. The isolates were identified after performing an array of biochemical tests in addition to molecular confirmation. Staphylococcus spp. and P. aeruginosa were found to be the predominating organisms isolated from diabetic wounds. The number of organisms isolated is given in Figure 1. The most important observation from the study is the isolation of V. parahaemolyticus from diabetic wounds. To the best of our knowledge, this is the first report in India to encounter V. parahaemolyticus in diabetic wounds.

Antibiotic susceptibility test

Antibiotic susceptibility test was performed for the confirmed 107 isolates. Susceptibility of bacterial isolates to the different

Fig. 1. Number of isolates recovered from diabetic wound

Fig. 2. Antibiotic susceptibility of bacteria isolated from diabetic wounds
2A: Susceptibility of Gram positive bacterial isolates; 2B: Susceptibility of Gram negative bacterial isolates other than P. aeruginosa; 2C: Susceptibility of P. aeruginosa
| Bacteria                      | Primer | Oligonucleotide sequence (5’–3’)         | Annealing temperature | Amplicon size (bp) |
|-------------------------------|--------|----------------------------------------|-----------------------|--------------------|
| *Staphylococcus*              | ST1    | GGC CGT GTT GAA CGT GGT CAA            | 55                    | 370                |
|                               | ST2    | ATC ATTA CTA TTT CAG TAC CTT CTG GTA A |                       |                    |
| *Staphylococcus aureus*       | SA1    | AAT CTT TGT CCG TAC ACG ATA            | 57                    | 104                |
|                               | SA2    | TTC TTC ACACGT AAT GAG ATT TCA GTA GAT AAT ACA ACA | 57 | 128 |
| *Staphylococcus epidermidis*  | SE1    | ATC AAA AAG TTG GCG AAC                | 56                    | 128                |
|                               | SE2    | CTT TTC ACA AAAG AGC AGC GTG GAG AAA AGT ATC A | 56 | 128 |
| *Vibrio*                      | rpoA1  | CGT AGC TAG AGG CAA AGA                | 55                    | 197                |
| *Acinetobacter*               | RpoB1  | CTG TCA TGA CCT GGA ACG               | 59                    | 940                |
|                               | RpoB2  | GAT ATCC AGG TAC TGA CCG ACG TTC AT   |                       |                    |
| *Citrobacter freundii*        | Cfa 1  | TTG GCG TCC AGC GCA TCC               | 57                    | 100                |
|                               | Cfa 2  | AAA TCC AGC CTT CGG CAA ACG           |                       |                    |
| *Escherichia coli*            | UIDA1  | AAA AGC GCA AGA AA AGC                | 63                    | 147                |
|                               | UIDA2  | AGACG CGT GGT TAC AGT CTT GCC         |                       |                    |
| *Klebsiella spp.*             | KpNM1  | ATT TGA AGA GGT GTC AAA               | 55                    | 130                |
|                               | KpNM2  | CGA TTT TCC ACT CCT AAG TTT TCT GTG GTC | 55 | 130 |
| *Vibrio parahaemolyticus*     | Tlh1   | AAAAACGATTATGCAAGAAGCAAGCTGGCT        | 55                    | 450                |
|                               | Tlh2   | ACTTTCTAGCATTTCCTCATGC                |                       |                    |
| *Pseudomonas aeruginosa*      | OprL1  | ATG GAA ATG CTT AAA TTC GGCC          | 55                    | 504                |
|                               | OprL2  | T CTT CAG CTC GAC GCG ACG             |                       |                    |
antibiotics used is shown in Figure 2. Among the Gram positive isolates, resistance for oxacillin was significantly high. Bacteria were found to be sensitive for tetracycline followed by vancomycin Figure 2A. Among the Gram negative isolates other than *P. aeruginosa*, resistance was significantly more to ampicillin and amoxicillin when compared to other antibiotics used Figure 2B. Around 60% of the isolates were sensitive to imipenem. In general, more number of isolates was found to be resistant to the antibiotics used. All isolates of *E. coli* were completely resistant to the antibiotics used other than tetracycline. *Klebsiella* spp. (94% isolates) showed highest resistance to amoxicillin. *Acinetobacter* spp. were highly sensitive to imipenem and resistant towards ampicillin and amoxyclov. Complete resistance was found to cefocitin/cloxacillin among the *Vibrio* spp. *P. aeruginosa* isolates were generally sensitive to all the antibiotics used Figure 2C. Nearly 40% of the isolates showed resistance to cefperazone/sulbactam combination. Around 85% of isolates showed least resistance to ciprofloxacin.

**Biofilm assay**

Out of 107 isolates checked for their biofilm forming abilities, 80 isolates formed black colonies with a dry crystalline consistency indicating a positive result. *Staphylococcus* spp. (88%) and *P. aeruginosa* (88%) were the predominant biofilm formers. 53% isolates of *Klebsiella* spp., 80% isolates of *Vibrio* spp. and 37% isolates of *Acinetobacter* spp. formed biofilm. Both the *E. coli* isolates were biofilm formers. In the microtitre plate assay, biofilm formation varied at different time intervals. High biofilm formers were found in *Staphylococcus* spp., *Klebsiella* spp. and *P. aeruginosa* isolates. Majority of the *Staphylococcus* spp. and *E. coli* isolates were moderate biofilm formers (92%). The numbers of high biofilm formers (9%) were more in *P. aeruginosa* when compared to other bacteria. High biofilm formers were not found in *E. coli,*
Vibrio spp. and Acinetobacter spp. Majority of the isolates formed weak and moderate biofilms at 24 h. P. aeruginosa isolates showed moderate biofilm formation at 24 h. But, at 48 h around 27% of them showed high biofilm formation. At 72 h, around 7% of *Klebsiella* spp. showed high biofilm formation. In *P. aeruginosa* the number of isolates forming weak biofilm increased at 72 h. In *E. coli*, Acinetobacter spp. and Vibrio spp. the number of isolates forming moderate biofilm increased at 48 and 72 h. There was significant association between incubation time and intensity of biofilm formation in *P. aeruginosa* ($\chi^2(p<0.05) = 0.001$), Staphylococcus spp. ($\chi^2(p<0.05) = 0.023$) and Acinetobacter spp. ($\chi^2(p<0.05) = 0.018$). There was no significant association in *Klebsiella* spp., *E. coli* and Vibrio spp. The percentage of biofilm formed at different time intervals is shown in Figure 3.

**DISCUSSION**

Diabetic foot infections are a major problem worldwide. In India, more than 62 million people have been diagnosed with diabetes. Foot ulcer is the major problem in diabetes which if left untreated, results in limb amputation\(^9\). In the present study, isolation and identification of bacteria causing foot ulcers along their antibiotic susceptibility profile and biofilm forming ability were attempted. As reported from the present study, the percent prevalence of Gram negative bacteria was more than the Gram positive bacteria. This corresponds with the previous study which also shows the predominance of Gram negative bacteria in diabetic wounds\(^20, 21\). A study from Malaysia has reported *Proteus* spp. to be the predominating organism in diabetic wound\(^22\). However, *Proteus* spp. was hardly encountered in this study. *S. aureus* and *P. aeruginosa* were the predominant organisms isolated and identified in this study. Contradicting results have been observed in a study which has shown the prevalence of *E. coli* in diabetic wounds\(^21\). The present study highlights presence of multidrug-resistant bacteria in diabetic wounds as depicted by its resistance to more than one drug used. Gram positive isolates showed resistance to vancomycin in our study. Contradictory results have been seen in a study which has shown 100% sensitivity of *S. aureus* to vancomycin\(^22\). Gram negative bacteria in the current study have shown significant resistance to amoxicillin-clavulanic acid. The results are in agreement with a study which has shown similar result\(^21\). Resistance to imipenem was around 30%. The results does not correspond with a study has shown 100% sensitivity of Gram negative bacteria towards imipenem\(^23\). Infections with bacteria forming biofilms are difficult to eradicate. These biofilms are not only less susceptible to host cell immune responses but also have a high tolerance to antibiotics than the planktonic cells\(^24\). The resistance of biofilm forming bacteria towards antibiotics is due to obstruction in the permeability of the drug by the polysaccharide matrix\(^25\) and alteration of the drug efficacy in the biofilm environment\(^1\). Not only biofilm effect antimicrobial agents, but also they give protection against host defenses. Biofilms have anti-phagocytic activity and also inactivates complement and antibodies\(^26\). In the present study, 75 per cent of drug resistant bacteria were biofilm formers. The percent of biofilm formers in our study is significantly larger in comparison to a previous study\(^21\) and corresponds to studies by Swarna et al. and James et al\(^27, 28\). The higher percentage of biofilm formers in diabetic wounds could be due to ineffective debridement procedure or longer duration of ulcer in patients\(^29\). *P. aeruginosa* was a predominant biofilm former with 89 per cent of the isolates being positive for biofilm formation. This was an expected result as studies have reported biofilm formation by *P. aeruginosa* more readily in diabetic wound environment\(^20\).

**CONCLUSION**

It is clear from the present study that, majority of bacteria isolated from diabetic wounds are multi-drug resistant and moderate-high biofilm formers which resist antibiotic therapy. In order to decrease the undesirable consequences associated with diabetic wounds, it is essential to recognize the biofilm forming abilities of the organism in addition to their antibiotic susceptibility profile. Decline in the morbidity due to diabetic foot ulcers caused by multidrug resistant biofilm producing bacteria is possible by adopting alternative therapies which prevent bacterial attachment, disrupt biofilm and
act as quorum sensing inhibitors. Developing new tools to reduce the suffering of diabetic patients with foot ulcers should be taken as a challenging research.

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