Identification of biofilm-producing microorganism’s and drug susceptibility pattern from diabetic foot ulcer patients at Puducherry

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ABSTRACT

Diabetes mellitus is a significant health problem worldwide that affects approximately 171 million people; severe complications lead to the development of diabetic foot ulcers. Diabetic ulcer infections are mainly polymicrobial in nature and multidrug-resistant (MDR), which is capable of forming a biofilm, which is the important virulence factor results in treatment failure. The main objectives of this study to investigate the etiologic agents of diabetic foot infections, their antimicrobial resistance and biofilm formation. A total of 200 patient samples were taken from diabetic foot ulcer patients between September 2015 and February 2016. Isolation and identification of microorganism were made according to standard microbiological procedures. AntibioticSusceptibility testing performed by Kirby Bauer disc diffusion method and the biofilm production was performed by the tube method and Congo Red Method. Out of 200 samples processed, 110 (55%) were polymicrobial, 50 (25%) monomicrobial and 40 (20%) culture Sterile. The most common organism isolated were 82 (39%) Pseudomonas aeruginosa, 45 (21%) Staphylococcus aureus, 48 (23%) Candida sp followed by others. Biofilm production were seen in 112 (53%) of the isolates. Antimicrobial drug resistance was higher among 92 (82%) biofilm producers than non-biofilm 20 (18%) producing microorganisms. Organisms isolated from chronic diabetic foot ulcers cases were multidrug-resistant and biofilm producers. Our study shows the importance of biofilm screening with the usual antibiogram, as a routine technique in diabetic foot ulcers patients for effective treatment.

INTRODUCTION

Chronic wounds in diabetic foot ulcer affect 1 - 2% of the population, persistent morbidity with delay in the wound healing process (Gjødsbol et al., 2006; Davis and Brown, 2016).

Biofilm is a structured consortium of bacteria and extracellular matrix, which is essential for interconnecting the bacteria and can be composed of polysaccharides, proteins, and extracellular DNA (Whitchurch, 2002).

Almost 50-60% of chronic wound specimens were characterised as biofilm containing, whereas only 6%-10% of acute wounds, biofilms were...
more prevalent in the chronic wound than acute wounds (Malone and Swanson, 2017; James et al., 2008).

Biofilm producing organisms exchange the genetic material which altered phenotype and delays the wound healing process (Murali et al., 2014). Biofilm is a crucial virulence factor as it creates a suitable environment for them to survive and evade antibiotics.

Pseudomonas aeruginosa, Staphylococcus aureus and Candida sp are the most causative microorganism in chronic wounds, as a biofilm producer, that delaying the wound healing process and responsible for resistance to antimicrobial therapy (Davis and Brown, 2016; Mantero et al., 2017). They are difficult to eradicate using antibiotics, identification of biofilm-producing organism among clinical isolates lead to better management of Diabetic Foot ulcer instead of antibiotic treatment failure (Schierele et al., 2009).

The aim of our present study to identify the microorganisms from diabetic foot ulcer patients and their antimicrobial susceptibility pattern and biofilm formation.

MATERIALS AND METHODS

This prospective study was conducted at the Department of Microbiology, in a tertiary care hospital, Puducherry during six months from September 2015 to February 2016. A total of 200 samples from Diabetic Foot ulcer patients attending the surgery outpatient department were included in this study. Institutional ethical clearance was taken, and informed consent was obtained.

Samples were collected using a sterile cotton swab from the deeper parts of the wound and sent to the laboratory. Samples were processed according to standard microbiological procedures. Antimicrobial susceptibility testing was performed by disc diffusion method according to the Clinical Laboratory and Standards Institute Guidelines.

Biofilm formation by tube method

A loopful of pure culture on Tryptone soya agar was inoculated into 2ml of Tryptone soy broth in Falcon tubes. The tubes were incubated for 24 hours at Phosphate buffered saline and left to dry. 2 ml crystal violet (1%) were added for 15 minutes to stain the Cells. Tubes were washed with water to remove the unbound dye for drying place the tubes in Inverted Position. Presence of a stained film on the sides of the tube was positive of biofilm formation. Biofilm formation was reported as strong, moderate or weak.

Congo Red method

Congo Red Plates were inoculated and incubated aerobically for 24–48 hours at 37°C. Biofilm forming bacteria shows.

Positive - Black colonies with a dry crystalline morphology

Negative - Pink, dark at the centre of the colony

Biofilm Production of Candida

Isolated organisms inoculated into a tube containing 10ml Sabouraud’s liquid medium with glucose. The tubes were incubated at 37°C after 24 hours broth was discarded and tubes walls were stained with safranin. Biofilm formation was scored as negative (0), weakly positive (1+), moderately positive (2+) and strongly positive (3+).

RESULTS

Total of 200 samples was collected from patients with diabetic foot ulcers, 124(62%) were male, and 76(38%) were females, age group ranges from 45-85 years.

Based on the microbial growth, most organisms isolated were Polymicrobial. In 200 samples collected, 110(55%) isolates were polymicrobial, and 50(25%) monomicrobial and 40(20%) were Culture Sterile shown in Table 1.

Table 1: Microbial Growth in DFU

| Microbial Growth | Total Cases | Percentage (%) |
|------------------|-------------|----------------|
| Polymicrobial    | 110         | 55%            |
| Monomicrobial    | 50          | 25%            |
| Culture Sterile  | 40          | 20%            |

Table 2: Isolated Microorganisms from Diabetic Foot Ulcer cases

| Microorganisms         | Number of Isolates (n=210) | Percentage (%) |
|------------------------|----------------------------|----------------|
| Pseudomonas aeruginosa | 82                         | 39%            |
| Staphylococcus aureus  | 45                         | 21%            |
| E. coli                | 18                         | 9%             |
| Other bacteria         | 17                         | 8%             |
| Candida sp             | 48                         | 23%            |

Among 160 Positive Diabetic Foot ulcer cases, 210 microbial isolates were isolated. The most common
Table 3: Drug-Resistant organisms

| Microorganism       | No of Drug-Resistant Organisms (n=210) | Percentage (%) |
|---------------------|----------------------------------------|----------------|
| Pseudomonas aeruginosa | 38(82)                                 | 46%            |
| Staphylococcus aureus  | 29(45)                                 | 64%            |
| E. coli              | 12(18)                                 | 67%            |
| Other bacteria       | 8(17)                                  | 47%            |
| Candida sp           | 15(48)                                 | 31%            |

Table 4: Biofilm Formation by tube method

| Biofilm | Total Isolates (n=210) | Percentage |
|---------|------------------------|------------|
| Positive | 112                    | 53%        |
| Negative | 98                     | 47%        |

Table 5: Biofilm Formation by Congo red method

| Biofilm | Total Isolates (n=210) | Percentage |
|---------|------------------------|------------|
| Positive | 116                    | 55%        |
| Negative | 94                     | 45%        |

Table 6: Biofilm Producing Microorganisms

| Microorganism       | Tube Method (n=112) | Congo Red Method (n=116) |
|---------------------|---------------------|--------------------------|
| Pseudomonas aeruginosa | 40(49%)              | 44(54%)                  |
| Staphylococcus aureus   | 30(67%)              | 28(62%)                  |
| E. coli              | 15(83%)              | 17(94%)                  |
| Other bacteria       | 11(65%)              | 11(65%)                  |
| Candida sp           | 16(33%)              | 16(33%)                  |

Microorganisms isolated were Pseudomonas aeruginosa 82(39%) followed by Staphylococcus aureus 45(21%), Escherichia coli 18(9%), Others 17(8%), Candida sp 48(23%) were isolated mentioned in Table 2.

Table 3 Shows among 210 isolates, high Antibiotic resistance was seen in 38 (46%) Pseudomonas aeruginosa, 29(64%) Staphylococcus aureus, E.coli 12(67%), Other bacteria 8(47%), Candida sp, 15(31%) followed by Others isolates.

Among 210 isolates tested for biofilm production by tube method 112 (53%) isolates were biofilm producers, 98(47%) isolates were non Biofilm shows in Table 4.

Out of 112 biofilm-forming isolates, 61(54.4%) were strong biofilm producer, 32(29%) were moderate biofilm producer, while 19 (17%) were weak biofilm Producer.

In Table 5 out of 210 isolates, the positive result was noted in 116(55%) isolates formed black colonies with a dry crystalline morphology, whereas 94 (45%) shows negative result. P. aeruginosa were the predominant biofilm producer followed by Staphylococcus aureus.

Biofilm production was positive for 112(53%) isolates by tube method, and 116(55%) isolates by Congo Red method. Commonly isolated pathogens, 15(83%)E. coli, 30(67%)Staphylococcus aureus, 40(49%) Pseudomonas aeruginosa, was the predominant strong biofilm producer followed by others presented in Table 6.

Out of 48 Candida species isolated, 32(67%) was found to be biofilm producers. Biofilm production was most frequent among non-albicans Candida 22(69%) than Candida albicans 10(31%). Among the non-albicans Candida species, C. parapsilosis12 (37.5%) was the highest biofilm producer.

Among 112 biofilm producers in our study, 92(82%) isolates which were biofilm producers were drug-resistant strains, whereas 20(18%) biofilm-forming isolates were Sensitive to all drugs.

DISCUSSION

Foot ulcer infection is the major public health problem in diabetes; if left untreated, it results in limb amputation (Singh et al., 2005). The main objective of the present study, to identify the microorganisms and their antimicrobial susceptibility profile and biofilm formation in diabetic foot ulcer patients.

A total of 200 patient samples with diabetic foot infections of both sexes, age between 35-80 years were examined through a period of six months. Among them, 36 (72%) were male, and 14 (28%) were females. Males were more infected in diabetic foot ulcer than females due to increased outdoor work, a similar study conducted by (Yerat and Rangasamy, 2015).

Based on the microbial growth, most organisms isolated were Polymicrobial in our study correlates with (Manisha et al., 2012) study.

Our study isolated 210 microbial isolates. The most common microorganism isolated were Pseudomonas aeruginosa 82(39%) followed by Staphy-
**lococcus aureus** 45(21%), *Escherichia coli* 18(9%), *Others* 17(8%), *Candida sp* 48(23%) were isolated correlates with the previous study which shows the predominance of Gram-negative bacteria in diabetic wounds (Rani and Nithyalakshmi, 2014; Banu et al., 2015).

In our Present study among 210 isolates, high Antibiotic resistance was seen in 38 (46%)*Pseudomonas aeruginosa*, 29(64%)*Staphylococcus aureus*, *E.coli* 12(67%), *Other bacteria* 8(47%), *Candida sp.*, 15(31%)followed by *Others*, similar study reports also showed the antibiotic-resistant pattern (Shankar et al., 2005; Raja, 2007).

Biofilm formation makes microorganisms much more resistant to antimicrobial therapy. It involves penetration of antibiotics through the biofilm matrix, reduced oxygen and nutrient, decreased growth rates and metabolism (Keren et al., 2004).

Among 210 isolates tested for biofilm production by tube method.112 (53%)isolates by tube method, and 116(55%) isolates by Congo red method were biofilm producers, 98(47%) isolates by tube method and 94(45%) isolates by Congo red method isolates were non Biofilm producers. These results Correlate with (Swarna et al., 2012) study.

Among all isolates *Pseudomonas aeruginosa, Staphylococcus aureus, E.coli* was the most strong biofilm producer in our study. Biofilm formation by *P. aeruginosa* more readily in the diabetic wound environment report by (Damir, 2011).

The antimicrobial resistance was higher in biofilm-producing isolates than in non-biofilm producers. Among 112 biofilm producers in our study, 92(82%) isolates which were biofilm producers were drug-resistant strains, whereas 20(18%) biofilm-forming isolates were Sensitive to all drugs.

Biofilm enhances the emergence of MDR in chronic foot ulcer infections. A drug-resistant pattern in biofilm formation correlates with a previous study (Banu et al., 2015).

**CONCLUSIONS**

Diabetic foot ulcer infections are problematic issues due to their polymicrobial nature, antimicrobial resistance, and biofilm formation. Our study suggests a routine screening test for biofilm production and antibiograms will help surgeons to formulate effective treatment options for these patients.

**Conflict of interest**

The authors declare that they have no conflict of interest for this study.

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**REFERENCES**

Banu, A., Rajkumar, H. M. M., Srinivasa, J., S 2015. A spectrum of bacteria associated with diabetic foot ulcer and biofilm formation: a prospective study. *Australas. Med. J.*, 8(9):280–285.

Damir 2011. Why do Diabetic Foot Ulcers not heal. *Journal International Medical Sciences Academy*, 24:205–205.

Davis, R., Brown, P. D. 2016. Multiple antibiotic resistance index, fitness and virulence potential in respiratory *Pseudomonas aeruginosa* from Jamaica. *Journal of Medical Microbiology*, 65(4):261–271.

Gjødsbøl, K., Christensen, J. J., Karlsmark, T., Jørgensen, B., Klein, B. M., Krogfelt, K. A. 2006. Multiple bacterial species reside in chronic wounds: a longitudinal study. *International Wound Journal*, 3(3):225–231.

James, G. A., Swogger, E., Wolcott, R., deLancey Pulcini, E., Secor, P., Sestrich, J., Costerton, J. W., Stewart, P. S. 2008. Biofilms in chronic wounds. *Wound Repair and Regeneration*, 16(1):37–44.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., Lewis, K. 2004. Persister cells and tolerance to antimicrobials. *FEMS Microbiology Letters*, 230(1):13–18.

Malone, M., Swanson, T. 2017. Biofilm-based wound care: the importance of debridement in biofilm treatment strategies. *British Journal of Community Nursing*, 22(Sup6):S20–S25.

Manisha, J., Mitesh, H. P., Dhara, N. K. S., Vegad, J., M 2012. A spectrum of microbial flora in diabetic foot ulcer and its antibiotic sensitivity pattern in tertiary care hospital in Ahmed Abad, Gujarat. Nation. *J. Med.*, 2(3):354–357.

Mantero, M., Gramegna, A., Pizzamiglio, G., D’Adda, A., Tarsia, P., Blasi, F. 2017. Once daily aerosolised tobramycin in adult patients with cystic fibrosis in the management of *Pseudomonas aeruginosa* chronic infection. *Multidisciplinary Respiratory Medicine*, 12(1).

Murali, T. S., Kavitha, S., Spoorthi, J., Bhat, D. V., Prasad, A. S. B., Upton, Z., Ramachandra, L., Acharya, R. V., Satyamoorthy, K. 2014. Characteristics of microbial drug resistance and its correlates in chronic diabetic foot ulcer infections. *Journal of Medical Microbiology*, 63(10):1377–1385.

Raja, N. S. 2007. *Microbiology of diabetic foot infections in a teaching hospital in Malaysia: a retrospective study of 194 cases. Journal of*
Microbiology-Immunology and Infection, 4(1):39–44.

Rani, V., Nithyalakshmi, J. 2014. A comparative study of Diabetic and Non-diabetic wound infections with particular reference to MRSA and ESBL. *Int. J. Curr. Microbiol. App. Sci.*, 3(12):546–554.

Schierle, C. F., la Garza, M. D., Mustoe, T. A., Galiano, R. D. 2009. Staphylococcal biofilms impair wound healing by delaying reepithelialization in a murine cutaneous wound model. *Wound Repair and Regeneration*, 17(3):354–359.

Shankar, E. M., Mohan, V., Premalatha, G., Srinivasan, R. S., Usha, A. R. 2005. Bacterial etiology of diabetic foot infections in South India. *European Journal of Internal Medicine*, 16(8):567–570.

Singh, N., Armstrong, D. G., Lipsky, B. A. 2005. Preventing Foot Ulcers in Patients With Diabetes. *JAMA*, 293(2):217–228.

Swarna, S. R., Madhavan, R., Gomathi, S., Thamarai-selvi, S. 2012. A study of Biofilm on Diabetic Foot Ulcer. *Int. J. Res. Pharm. Biomed. Sci.*, 3:1809–1814.

Whitchurch, C. B. 2002. Extracellular DNA Required for Bacterial Biofilm Formation. *Science*, 295(5559):1487–1487.

Yerat, R., Rangasamy, V. 2015. A clinicomicrobial study of diabetic foot ulcer infections in South India. *International Journal of Medicine and Public Health*, 5(3):236–236.