



## Screening and Characterization of Biofilm Formation by *Staphylococcus* Spp and *Pseudomonas* Spp Isolates from Diabetic Foot Ulcer

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**Abstract:** Diabetes mellitus is a very serious systemic disease worldwide. Around 416 million cases were estimated in 2015 worldwide, and are expected to reach 549 million in 2030 which is 8.6% higher. Diabetes mellitus is the leading cause of mortality and morbidity and is responsible for 3.8 million deaths annually. 25% of the diabetic patients are affected with foot infections out of which, 15% people are forced for limb amputation which affects the quality of life of the patients. Diabetic foot ulcer is a poly microbial infection mostly occurs due to *Staphylococcus* spp and *Pseudomonas* spp and pose serious complications. Bacteria are the cause for much type of diseases and generate resistance to wide range of biofilm forming. Biofilms constitute reservoirs of pathogens and are associated with resistance to antimicrobial agents and chronic infection. The study included 156 patients (59% male and 41% female) suffering diabetic foot ulcer whose pus culture was isolated. The identification of isolates for both gram-negative and gram-positive organisms was done as per the procedures mentioned in Bergey's manual of Determinative Bacteriology. Further, MALDI- TOF (Matrix-Assisted Laser Desorption/Ionization) was used to confirm the identification of the isolates using classical methods. *Staphylococcus* spp (65%) and *Pseudomonas* spp (35%) biofilm producing isolates were identified for Congo red method assay and Tissue culture plate method. Results of biofilm production in positive, intermediate, negative differentiation on the Congo red plate assay and Tissue culture plate method assays were analyzed.

**Keywords:** Diabetic Foot Ulcer, Diabetic Foot Infection, Biofilm, *Staphylococcus* spp, *Pseudomonas* spp.

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Received On 26 June, 2021

Revised On 30 August, 2021

Accepted On 8 September, 2021

Published On 16 September, 2021

**Funding** This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

**Citation** Venkatesan.R, Suresh S S Raja, Vijayakumar.R, Panneer Selvam.K , Screening and characterization of biofilm formation by *Staphylococcus* and *Pseudomonas* species isolated from diabetic foot ulcer.(2021).Int. J. Life Sci. Pharma Res.11(5), 139-144  
<http://dx.doi.org/10.22376/ijpbs/lpr.2021.11.5.L139-144>

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Int J Life Sci Pharma Res., Volume 11., No 5 (September) 2021, pp 139-144

## 1. INTRODUCTION

Worldwide diabetes mellitus is a very serious systemic disease with higher incidence in the United States and more than 30 million diabetes mellitus patients suffer multisystem organ involvement frequently.<sup>1</sup> Diabetes mellitus is the leading cause of death of 3.8 million annually.<sup>2</sup> Diabetes mellitus is associated with long time complications of mortality or morbidity among which foot ulceration is a major problem that affects the quality of life.<sup>3</sup> The peripheral vascular disease, neuropathy, foot ulceration and infection in several pathological complications leads to gangrene development that necessitates limb amputation. There is a greater risk of being hospitalized for soft tissue and bone infections.<sup>4</sup> Annually, the incident of foot ulceration is 1.0-4.1% and prevalence is 4-10%. The principle causative factors are peripheral neuropathy, vascular compromise, ulceration and infections.<sup>5</sup> The foot infections are common and serious problem in person with diabetes. Patients having impaired microvascular circulation have limited access of phagocytes in diabetic foot ulcer.<sup>6</sup> India is called the diabetes capital of the world, with the world's largest number of diabetic disorders.<sup>7</sup> The commonest devastating complication in diabetic patients is the non traumatic lower limb amputation which is due to diabetic foot ulcer (8% to 17% in India)<sup>8</sup> and diabetic foot infections. Diabetic foot infection (DFI) occurs predominantly due to a polymicrobial combination of gram positive bacteria like *Staphylococcus aureus* or *Enterococcus* and gram negative like *Pseudomonas aeruginosa*, *E.coli*, *Klebsiella pneumoniae* and *Proteus* species etc.<sup>9</sup> The microorganism that colonizes on the wound surface also provides an ideal niche for further invasion resulting in the infection. The interesting factor is that different microorganisms can exist independently or combined from micro-communities within a matrix of extracellular polymeric substances called as biofilm. The biofilm is denoted as a solid, robust layer of mucilage adhering to a surface and containing a community of bacteria and other microorganisms. The hydrophobicity of the bacteria plays an important role in the ability to form biofilms. The ability of a microorganism in forming the biofilms is an important virulence factor as it establishes a protective environment for the organisms to survive.<sup>10</sup> Biofilms pave the way for the re-emergence of multi-drug resistant strains. The study of diabetic foot ulcer and the isolated organisms helps to detect the biofilm formation among the bacteria.

## 2. MATERIALS AND METHODS

This study was conducted as a prospective study in 156 patients with diabetic foot ulcer, attending the inpatient and outpatient department, Government Hospital, Perambalur, Tamilnadu, India. The informed consent was obtained from the subjects in their own language. All patients above 20 years and below 70 years of age having chronic diabetic foot ulcer for a duration more than 3 months were included in the study. Patients history was collected with detailed demographic data including the age, sex, occupation, socio economic status and the types of treatment. The ulcers were graded using Wagner's grading 0 to V.

### 1.1. Inclusion Criteria

The patients of 20 to 70 year age, diabetic foot infection and foot ulcer of maximum three months and other disease and disorder based sample collection.

### 1.2. Exclusion Criteria

Non diabetic foot ulcer and foot infection were excluded from.

### 1.3. Wagner's grading 0 to V classification

Diabetic foot ulcers were given respective gradings as follows: Grade 0 – No open lesion, Grade I – Superficial ulcer, Grade II – Probing to tendon or capsule, Grade III – Deep ulcer with osteomyelitis, abscess and joint sepsis, Grade IV – Local gangrene in front foot whole ulcer, Grade V – Gangrene of entire foot. In the study, patients were mostly in Grade III contamination. The foot wounds and tissue debris were cleaned thoroughly with sterile water slowly with gentle rubbing of the DFI wound site and swab in with 70% of alcohol. The ulcers were assessed by the surgeons and culture material was collected with cotton tipped sterile swabs from the deeper parts of diabetic foot ulcer (Figure 1). The swab was deeply extended into the wound site majorly to avoid contamination from the surroundings. The copious volume of pus was aseptically collected by needle aspiration method to avoid the major exogenous contamination. The sample was then immediately transferred to sterile tube with peptone or glucose water, properly labeled and transported in aseptic condition within 24 hours to the laboratory.



**Fig.1. Chronic diabetic foot ulcer**

#### 1.4. Identification and Isolation

The sample was identified and isolated using specifically selected media of Mannitol salt agar and Cetrimide agar. For gram positive organisms such as *Staphylococcus* spp Mannitol salt agar media was used while, Cetrimide agar media was used for the isolation and identification of *Pseudomonas* spp. The culture was preserved and further experiments were carried out using Brain Heart infusion Agar (BHIA) and Nutrient Agar (NA). All the isolates were first subjected to the classical methods of identification like morphology, gram stain reaction, Biochemical reaction for Oxidase and Catalase according to the Brgey's manual of determinative bacteriology. Then all the identified isolates were confirmed further with Matrix-Assisted Laser Desorption/ Ionization (MALDI-TOF). All the 167 isolates (*Staphylococcus* spp 65% and *Pseudomonas* spp 35%) were identified for biofilm formation by Congo red method assay and tissue culture plate method.

#### 1.5. Congo Red Plate Assay

Biofilm formation was detected by Congo red method using a specifically prepared medium composed of brain heart infusion (BHI) broth (37gms/L), sucrose (50gms/L), agar no.1 (10gms/L) and Congo red stain (0.8gms/L). The Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes. Brain heart infusion (BHI) medium was separately prepared and autoclaved. Then agar medium was autoclaved at 55°C and cooled. Congo red stain was added to the cooled plates. The plates were then inoculated and incubated aerobically for 24 to 48 hours at 37°C. The plates were checked for biofilm formation. It was observed that the biofilm formers

produced black colonies with a dry crystalline consistency while weak slime producers usually remained pink, through occasional darkening at the centers of colonies. Indeterminate results were characterized by darkening of the colonies, with the absence of a dry crystalline colonial morphology (Figure 2 and 3). The tests were done in triplicates and repeated three times.<sup>11, 12</sup>

#### 1.6. Tissue Culture Plate Method

A sterile 96 well flat bottomed polystyrene microtiter plate was used for the tissue culture plate method. 230 µl of Trypticase Soy Broth (TSB) was taken and 20µl of overnight bacterial culture was added to the corresponding well of each strain. The negative control wells contained broth only. The plates were incubated aerobically for 24 hours at 35°C. The content of the wells was poured off and the wells were washed three times with 300µL of sterile distilled water. The bacteria adhering to the wells were fixed with 250µL methanol for 15 minutes. Then the wells were stained with 250µL of 1% solution of crystal violet for five minutes. Excess stain was removed by washing and wells were air dried. The dye bound to the wells was soluble with 250µL of 33 percent glacial acetic acid. The optical density (O.D) of each well was measured at 490nm to 600nm using an ELISA auto reader. The tests were carried out in triplicate and the result averaged. The OD was determined as three standard deviation points above the mean O.D of the negative control. The strains were classified as biofilm producers and non producers. The data was analyzed using Microsoft Excel 2010 Edition.<sup>13</sup> Those two methods were used to identify and isolate the biofilm producing strain. (Figure 3).



**Fig 2: Biofilm formation assay a) Tissue culture plates b) Congo red plate assay**

### 3. RESULTS

The study included 156 samples collected from diabetic foot ulcer patients (59% male patients and 41% female patients). The age ranged between 30 to 80 years. 167 isolates were identified using Congo red method assay for bio film

formation. (Table I), which showed positive 42%, moderate 14%, negative 44% for the gram positive strains and the same assay in gram negative strains *Pseudomonas* spp processed in positive 48%, moderate 15%, negative 37% (Table I).

Table 1: Congo Red Assay and Tissue Culture Plate Assay		
Congo red assay	<i>Staphylococcus</i> spp	<i>Pseudomonas</i> spp
Positive	42%	48%
Moderate	14%	15%
Negative	44%	37%
Tissue culture plate assay	<i>Staphylococcus</i> spp	<i>Pseudomonas</i> spp
Positive	19%	19%
Moderate	14%	18%
Negative	67%	63%

The tissue culture plate assay method in the gram positive strains *Staphylococci* spp, showed positive 19%, moderate 14%, negative 67% and gram negative strains of *Pseudomonas* spp were positive 19%, moderate 18%, negative 63 %. The overall study of 167 cultures showed positive 32%, moderate 15% and negatives 53%. (Table I).

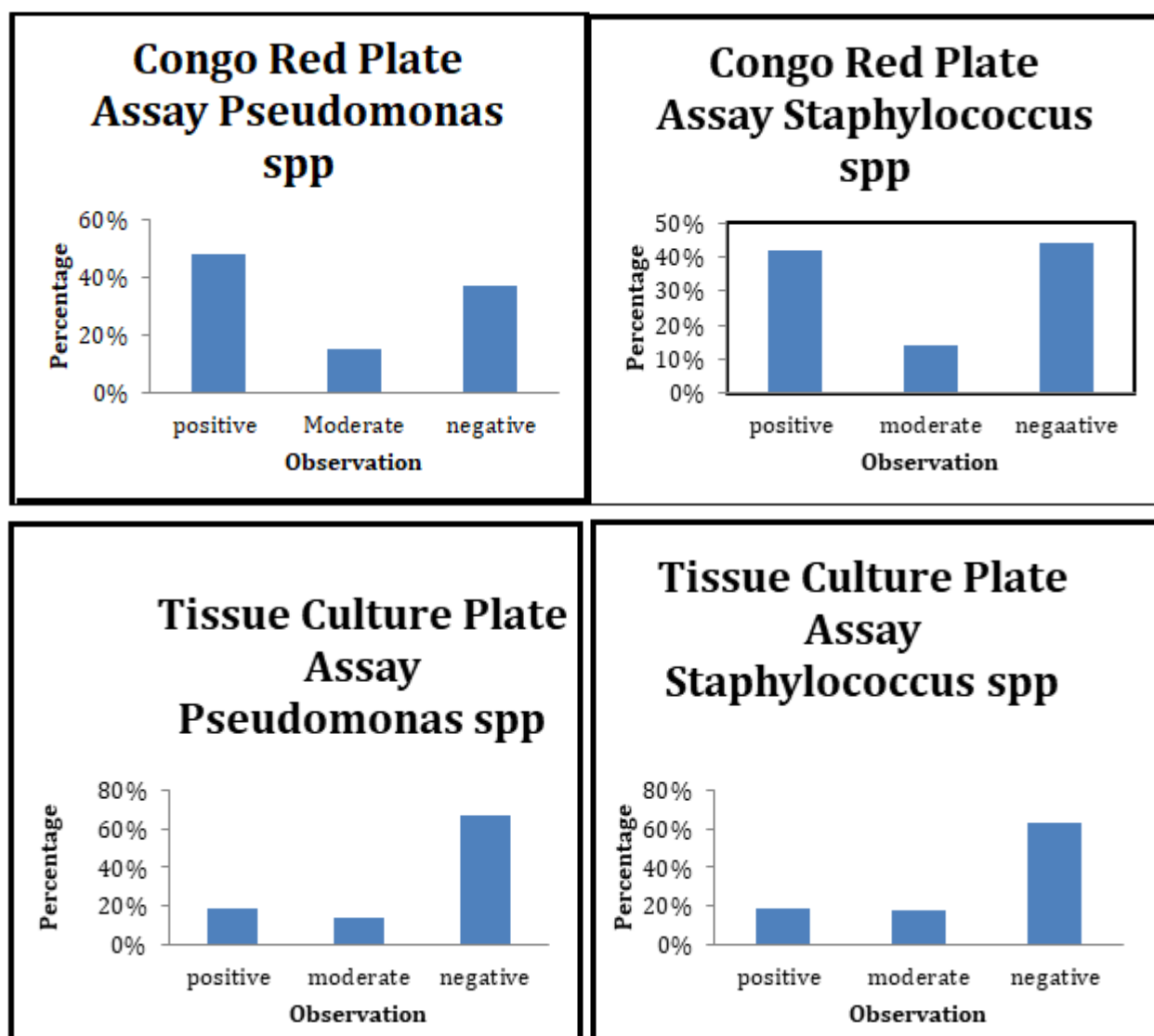


Fig 3: Congo red and tissue culture plate assay

#### 4. DISCUSSION

The host immune response in the *Staphylococcus* spp gives the ability of biofilm formation in chronic cases. Bacterial cells in the biofilm exhibit intrinsic resistance to antibiotics due to mechanism conferred by changes in the biofilm environment and inactivation of antimicrobial agents by exopolysaccharide.<sup>15</sup> The wound infection, healing and ischemia are the most common reasons for diabetes related amputation and 80% of lower limb amputations in diabetic patients are due to the biofilm formation in foot ulcer<sup>16,17</sup>. The diabetic mice showed that deleterious effect on the

innate immunity could lead to skin and soft tissue infections by *Staphylococcus* spp<sup>18</sup>. This is a common complication of these ulcers and infections, which if left untreated, results in limb amputation. According to United state national institute of health, more than 80% of chronic bacterial infections were associated with biofilms<sup>19</sup>. The main characteristic of DFU is the polymicrobial content that modulates bacterial virulence in within DFU microorganisms from a complex polymicrobial biofilm community and intercommunicate<sup>20</sup>. The present study includes samples of monomicrobial and polymicrobial isolates of DFU. The monomicrobial nature of infection is associated with the duration of the ulcer



treatment. In the early stages of infection, monomicrobial prevails and as infection progresses with time a polymicrobial in necrosis tend to emerge out.<sup>21</sup> The most studied bacterial interaction in diabetic foot ulcer is the cooperation between *Staphylococcus* spp and *Pseudomonas* spp formed in deeper wound bed<sup>22</sup>. There are many substances produced in *Pseudomonas* spp and *Staphylococcus* spp which play protective role against antibiotics<sup>23,24</sup>. The biofilm structure was analysed microscopically and biochemically that showed multiple layers of bacteria encased in a biofilm matrix containing proteins and polysaccharides. The mechanism for multidrug resistance in biofilm forming organisms is believed to be a direct result of close cell-cell contact in the biofilm which allows easy transfer of plasmids containing MDR genes amongst one another<sup>25</sup>. Organisms from the biofilm were also characterised with tolerance which is temporary and non heritable. Antibiotic mechanism of action depends on the division of cells which are inactive against microbes in biofilm and seems slow growing<sup>26</sup>. Biofilm play a vital role in delayed wound healing of DFU and DFLs. They are difficult to treat especially by using the classical antibiotics for preventing diffusion from biofilms. These biofilms encountered chronic wounds, such as diabetic foot ulcer are polymicrobial, which can enhance bacterial interaction. Thus, treatment methods are crucially needed, particularly because of the MDR bacteria.<sup>27</sup>

## 5. CONCLUSION

The chronic diabetic foot infection associated with biofilm formation has been concerned as a major challenge to diabetes treatment. In our study, we found that the biofilm producing bacteria showed more resistance to the antibiotics than non-biofilm producing bacteria. The study of biofilm formation by assay method is an easy and cost effective test that can be performed routinely in the laboratory. The biofilm detection will be helpful in effectively managing the infections in diabetic foot ulcer patients.

## 6. AUTHORS' CONTRIBUTIONS

Suresh SS Raja designed and supervised the experiments. Ramasmy Venkatesan carried out the research work and wrote the manuscript. R.Vijayakumar and K.Panneer Selvam interpreted the results and verified the final manuscript. All authors read and approved the final manuscript.

## 7. CONFLICT OF INTEREST

Conflict of interest declared none

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