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

Ramya Premanath[1]*, Sarika Suresh[1], Prathiksha P. Alva[1] and Akash S.K[2]

[1]Division of Infectious Diseases, Nitte University Centre for Science Education and Research, Paneer Campus, Deralakatte, Mangaluru- 575018, India.
[2]Kasturba Medical College, Hampankatta, Mangaluru – 575001, India.
*Corresponding author E- mail: ramya@nitte.edu.in

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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*, *Esherichia*, *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* [χ^2 ($p < 0.05$) = 0.001)], *Staphylococcus* spp. [χ^2 ($p < 0.05$) = 0.023)] and *Acinetobacter* spp. [χ^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.

Diabetes mellitus (DM), a metabolic disorder is increasing at an alarming rate all over the world. India has nearly 33 million diabetic subjects today. It tops the list of countries with the highest number of diabetics and considered the "diabetic capital of the world"[1]. The number of diabetics in India is expected to rise to a whopping 79.4 million by 2030 [2]. Diabetics are more susceptible to infections due to increased glucose levels and suppressed immune response as well as decreased blood flow to extremities that lead to slow healing wounds[3]. Diabetic foot infections caused by different genera of bacteria are the most feared complications of diabetes associated with high morbidity which can end up with gangrene and amputation. High incidence of diabetic foot infection in India can be attributed to practices such as bare foot walking, inadequate facilities for diabetic care, illiteracy and low socioeconomic status[4]. As bacteria that cause diabetic foot infection have become resistant to a number of available antibiotics, the most successful strategies to manage infection is the frequent debridement of foot ulcer[5]. Many of the organisms that cause

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[6]. 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[7]. 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 pathogens.

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[17]. Antibiotics norfloxacin, imipenem, tetracycline, gentamicin, amoxyclav, ampicillin, amoxicillin, ciprofloxacin, cefoxitin, cefotaxime were used for Gram negative bacteria, vancomycin, penicillin G, amoxyclav, 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[18] 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 37º C. 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 per cent 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 (OD_{600} 0.071 – 0.142), moderate biofilm formers (OD_{600} 0.142 – 0.284) and high biofilm formers ($OD600 \geq 0.284$).

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

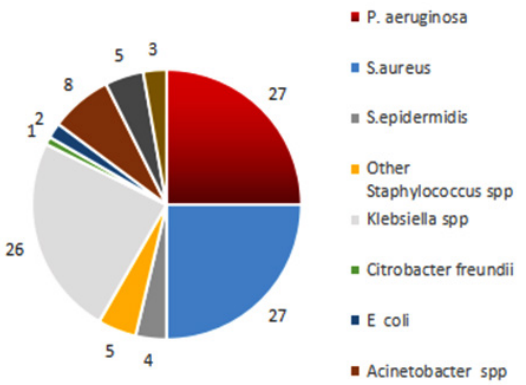

Fig. 1. Number of isolates recovered from diabetic wound

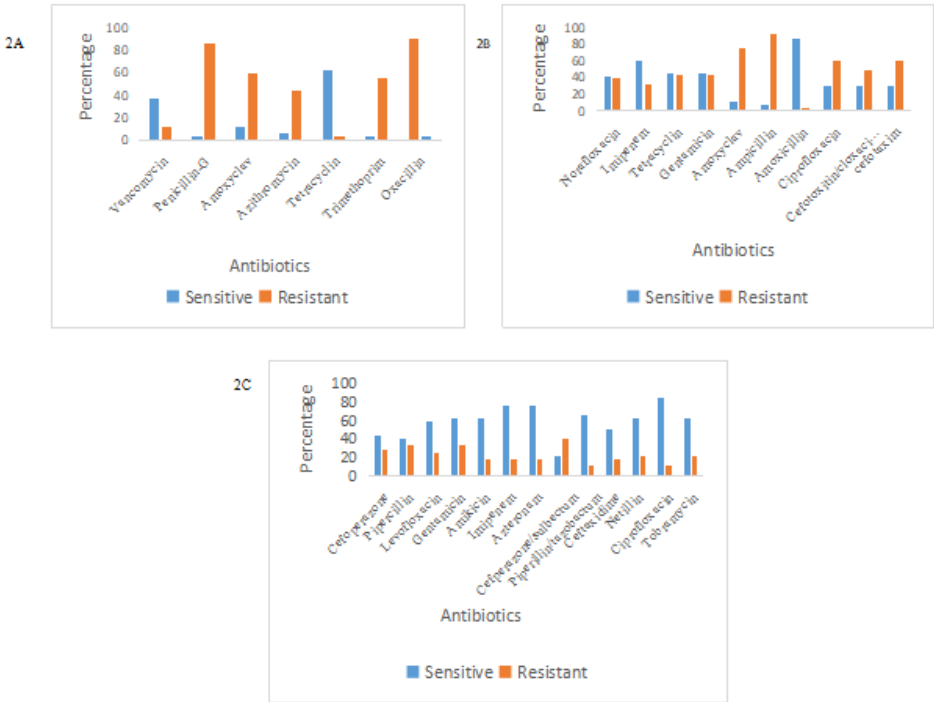

Fig. 2. Antibiotic susceptibility of bacteria isolated from diabetic wounds
2A: Susceptibility of Gram positive bacterial isolates; 2B: Susceptibility of Gram negative bacterial isolates other than *P.aeruginosa*; 2C: Susceptibility of *P. aeruginosa*

Table 1. Name and sequence of primers used for identification of different bacteria

Bacteria	Primer	Oligonucleotide sequence (5'–3')	Annealing temperature	Amplicon size (bp)
Staphylococcus[8]	ST1	GGC CGT GTT GAA CGT GGT CAA	55	370
	ST2	ATC ATTA CCA TTT CAG TAC CTT CTG GTA A		
Staphylococcus aureus[9]	SA1	AAT CTT TGT CGG TAC ACG ATA	57	104
	SA2	TTC TTC ACGCGT AAT GAG ATT TCA GTA GAT AAT ACA ACA		
Staphylococcus epidermidis [9]	SE1	ATC AAA AAG TTG GCG AAC	56	128
	SE2	CTT TTC ACAA AAG AGC GTG GAG AAA AGT ATC A		
Vibrio [10]	rpoA1	CGT AGC TAG AGG CAA AGA	55	197
	rpoA2	TGAAAG CTG GAA CAT AAC CAC GAA		
Acinetobacter[11]	RpoB1	CCT TCA TGA CCT GGA ACG	59	940
	RpoB2	GAT ATCC AGG ATC TGA CCG ACG TTC AT		
Citrobacterfreundii[12]	Cfa 1	TTG GCG TCC AGC GCA TTC	57	100
	Cfa 2	AAAT TCC AGC CTT CGG CAA ACG		
Escherichia coli [13]	UIDA1	AAA ACG GCA AGA AA AGC	63	147
	UIDA2	AGACG CGT GGT TAC AGT CTT GCG		
Klebsiella spp. [14]	KpNM1	ATT TGA AGA GGT TGC AAA	55	130
	KpNM2	CGA TTTC ACT CTG AAG TTT TCT TGT GTT C		
Vibrio parahaemolyticus[15]	Tlh1	AAAGCGATTATGCAGAAGCACTGGCT	55	450
	Tlh2	ACTTTCTAGCATTTTCTCTGC		
Pseudomonas aeruginosa [16]	OprL1	ATG GAA ATG CTG AAA TTC GGCCT	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 amoxyclav. 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*,

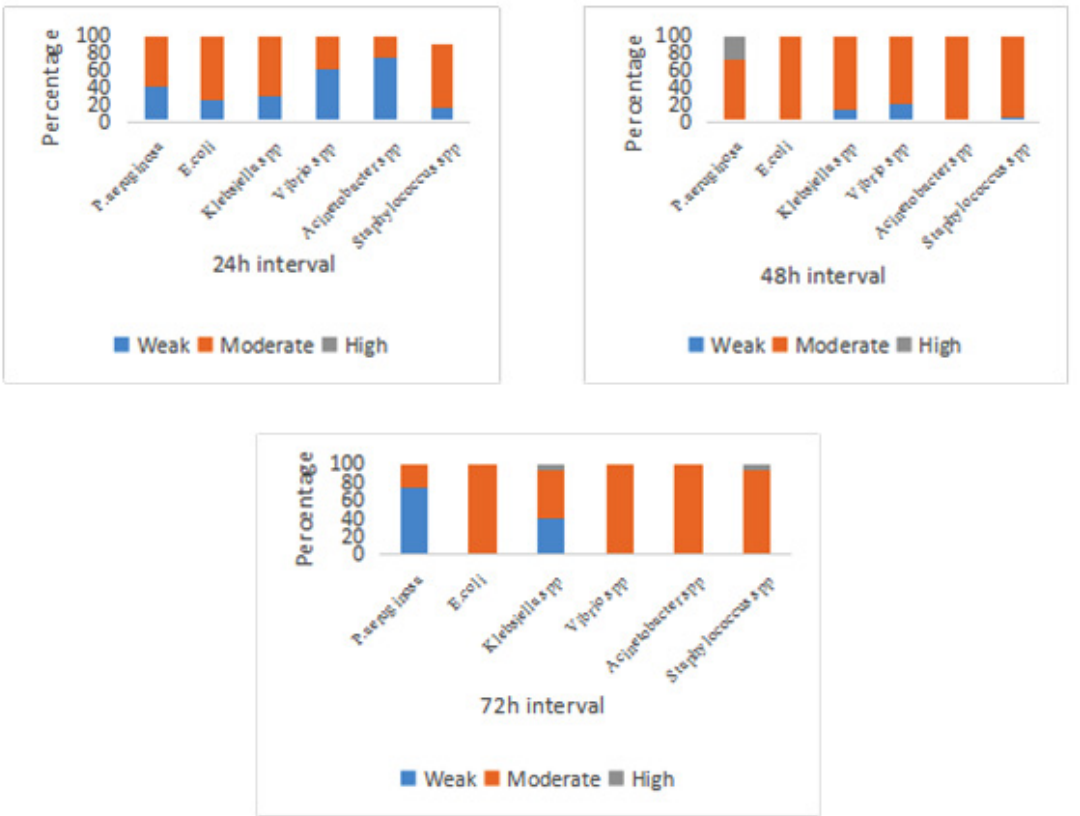

Fig. 3. Biofilm formation in the bacterial isolates at different time intervals (24,48 & 72 h)

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*, *Acientobacter* 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* [χ^2 (p< 0.05) = 0.001)], *Staphylococcus* spp. [χ^2 (p< 0.05) = 0.023)] and *Acinetobacter* spp. [χ^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[19]. 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[3]. 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[30].

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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REFERENCES

1. Murugan S, Mani K. R and Devi U. Prevalence of Methicillin Resistant *Staphylococcus aureus* among diabetic patients with foot ulcer and their antibiotic susceptibility patterns. *J. Clin. Diagn. Res*., **2**: 979-984 (2008).
2. Ramachandran A. Epidemiology of diabetes in India-three decades of research. *J. Assoc. Physicians India.*; **53**: 34-38 (2005).
3. Trivedi U, Paremeswaran S, Armstrong A, Burgueno-Vega D, Griswold J, Dissanaike S*et al*. Prevalence of multiple antibiotic resistant infections in diabetic versus non diabetic wounds. *J. Pathog.*, ID 173053 (2014).
4. Viswanathan V, Thomas N, Tandon N, Asirvatham A, Rajasekar S, Ramachandran A *et al*. Profile of diabetic foot complications and its associated complications – A multi centric study from India. *J. Assoc.Physicians India.*; **53**: 933-936 (2005).
5. Dowd SE, Wolcott R.D, Sun Y, Mc Keehan T, Smith E, Rhoads D. Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). *PloS One*. **3**: e3326 (2008).
6. Schierle C.F, De la Garza M, Mustoe T.A, Galiano R.D. Staphylococcal biofilms impair wound healing by delaying re epithelialization in a murine cutaneous wound model. *Wound Repair Regen.*, **17**: 354-359 (2009).
7. Murali T.S, Kavitha S, Spoorthi J, Bhat D.V, Prasad A.S, Upton Z *et al.*Characteristics of microbial drug resistance and its correlates in chronic diabetic foot ulcer infections. *J. Med. Microbiol*., **63**: 1377-1385 (2014).
8. Martineau F, Picard F.J, Ke D, Paradis S, Roy P.H, Ouellette M, *et al*. Development of a PCR assay for identification of staphylococci at genus and species levels. *J. Clin. Microbiol*., **39**: 2541-2547 (2001).
9. Martineau F, Picard F.J, Lansac N, Ménard C, Roy P.H, Ouellette M, *et al*. Correlation between the Resistance Genotype Determined by Multiplex PCR Assays and the Antibiotic Susceptibility Patterns of *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob. Agents. Chemother*., **44**: 231-238 (2000).
10. Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P. Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from in vivo work. *ISME J*., **2**: 19 (2008).
11. Wang J, Ruan Z, Feng Y, Fu Y, Jiang Y, Wang H, *et al.,* Species distribution of clinical *Acinetobacter* isolates revealed by different identification techniques. *PLoS One*.: e104882 (2014).
12. Kaclíková E, Krascsenicsová K, Pangallo D, Kuchta T. Detection and quantification of *Citrobacterfreundii* and *C. braakii* by 52 -nuclease polymerase chain reaction.*Curr. Microbiol*., **51**:229-232 (2005).
13. Bej A.K, Dicesare J.L, Haff L, Atlas R.M. Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for *uid*. *Appl. Environ. Microbiol*., **57**: 1013-1017 (1991).
14. Lee S.S, Chen Y.S, Tsai H.C, Wann S.R, Lin H.H, Huang C.K, *et al.,* Predictors of septic metastatic infection and mortality among patients with *Klebsiellapneumoniae* liver abscess. *Clin. Infect. Dis*., **47**: 642-650 (2008).
15. Bej A.K, Patterson D.P, Brasher C.W, Vickery M.C, Jones D.D, Kaysner C.A. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tlh, tdh and trh. *J. Microbiol. Methods.,* **36**:215-225 (1999).
16. Salman M, Ali A, Haque A. A novel multiplex PCR for detection of *Pseudomonas aeruginosa*: A major cause of wound infections. *Pak. J. Med. Sci*., **4**: 957 (2013).
17. Bauer A.W, Kirby W.M, Sherris J.C, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Path*., **45**: 493 (1966).
18. Freeman D.J, Falkiner F.R, Keane C.T. New method for detecting slime production by coagulase negative staphylococci.*J. Clin. Pathol*., **42**: 872–874 (1989).
19. Singh N, Armstrong D.G, Lipsky B.A. Preventing Foot Ulcers in patients with Diabetes. *JAMA.*, **293**:217–228 (2005).
20. Rani V,Nithyalakshmi J.A comparative study of Diabetic and Non-diabetic wound infections with special reference to MRSA and ESBL. *Int. J. Curr. Microbio.l App. Sci.*, **3**: 546–554 (2014).
21. Banu A, Hassan M.M, Rajkumar J, Srinivasa S. Spectrum of bacteria associated with diabetic foot ulcer and biofilm formation: a prospective study. *Australas. Med. J*., **8**: 280-285 (2015).

22. Raja NS. Microbiology of diabetic foot infections in a teaching hospital in Malaysia: a retrospective study of 194 cases. *J.Microbiol. Immunol. Infect*., **14**: 45–49 (2007).
23. Halpati, A., Desai, K. J., Jadeja, R., andParmar, M.A study of aerobic and anaerobic bacteria in diabetic foot ulcer and in vitro sensitivity of antimicrobial agent. *Int. J.Med. Sci. Public Health*., **3**: 818–821 (2014).
24. Ghafoor A, Hay D.I, Rehm A.H.B. Role of exopolysaccharides in *Pseudomonas aeruginosa* biofilm formation and architecture. *Appl. Environ. Microbiol*., **77**: 5238-5246 (2011).
25. Esmat, M. M, andSaif Al Islam,Diabetic foot infection: Bacteriological causes and antimicrobial therapy*J. Am. Sci*.; **8**: 389–393 (2012).
26. Leid, J. G, Shirtliff, M. E, Costerton, J. W, and Stoodley, P. Human leukocytes adhere to,penetrate, and respond to*Staphylococcus aureus* biofilms. *Infect. Immun*., **70**: 6339–6345 (2002).
27. James, G. A, Swogger, E, Wolcott, R, Pulcini, D.E, Secor, P, Sestrich, J., *et al* Biofilms in Chronic wounds. *WoundRepairRegen*., **16**:37–44 (2008).
28. Swarna, S. R, Madhavan, R, Gomathi, S, and Thamaraiselvi, S. A study of Biofilm on Diabetic Foot Ulcer. *Int. J. Res. Pharm. Biomed. Sci.*, **3**:1809–1814 (2012).
29. Zubair, M, Malik, A, Ahmad, J, Rizvi, M., Farooqui, K. J, and Rizvi, M. W. A study of biofilm production by gram-negative organisms isolated from diabetic foot ulcer patients. *Biol. Med.*, **3**:147–157 (2011).
30. Damir, A. Why Diabetic Foot Ulcers do not heal? *Journal International Medical Sciences Academy*., **24**: 205 (2011).